

***IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES***

Applicant: Rajiv SHAH, et al.

Title: METHOD FOR FORMULATING A GLUCOSE OXIDASE
ENZYME WITH A DESIRED PROPERTY OR PROPERTIES
AND GLUCOSE OXIDASE ENZYME WITH THE DESIRED
PROPERTY

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Examiner: Yong D. Pak

Art Unit: 1652

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APPEAL BRIEF UNDER 37 C.F.R. § 41.37

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Commissioner for Patents
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Alexandria, VA 22313-1450

Sir:

Under the provisions of 37 C.F.R. § 41.37, this Appeal Brief is being filed with the appropriate appeal fee under 37 C.F.R. 41.20(b)(2). An appeal fee of \$500.00 was submitted with Applicant's Appeal Brief dated December 7, 2005. In the Office Action dated March 8, 2006, the Examiner re-opened prosecution and stated that the previously paid notice of appeal fee and appeal brief can be applied to a new appeal (Office Action dated March 8, 2006, pg. 2, ll. 9-10.) Accordingly, Applicant requests that the previously paid fee for filing the Appeal Brief dated December 7, 2005, be applied to the present Appeal Brief. If that fee is deemed to be insufficient, authorization is hereby given to charge any deficiency (or credit any balance) to the undersigned deposit account 50-0872.

This communication is an Appeal Brief, responsive to the Final Office Action dated November 1, 2006, concerning the above-referenced patent application.

I. Real Party In Interest

The real party in interest for the above referenced patent application and the present Appeal is the assignee of record for the above referenced patent application, Medtronic Minimed, Inc., as recorded at Reel 012818, Frame 0025.

II. Related Appeals And Interferences

Applicant is not aware of any related appeals, interferences or legal proceedings that would have a bearing on the Board's decision in the present Appeal. However, Applicant filed a Notice of Appeal from a previous Final Office Action dated July 12, 2005, and filed a corresponding Appeal Brief on December 7, 2005. Following those filings, the Examiner reopened prosecution and issued a new Office Action in which certain rejections raised in the July 12, 2005, Final Office Action were withdrawn and certain new grounds of rejections were raised.

The present patent application claims the priority filing date of U.S. Provisional Application No. 60/335,585 (now expired), for which no substantive examination on the merits was conducted by the U.S. Patent and Trademark Office.

III. Status Of The Claims

Claims 1, 3-8, 10-46 and 48-54 are pending in the application.

However, claims 25-43 and 48-54 have been withdrawn from consideration by the Examiner.

Accordingly, claims 1, 3-8, 10-24 and 44-46 are pending and under consideration in the present application. Each of those claims is included in at least one of the rejections under general grounds identified in the Final Office Action and discussed in Sections VI. and VII., below.

The present appeal relates to each of the rejections and, thus, all of the rejected claims (i.e., claims 1, 3-8, 10-24 and 44-46).

IV. Status Of Amendments

No amendments have been filed, subsequent to the Final Office Action of November 1, 2006.

V. Summary Of Claimed Subject Matter

Embodiments of the present invention relate, generally, to a method employing directed evolution techniques for formulating a glucose oxidase enzyme having peroxide-resistant characteristics for use, by way of example, in a sensing device.

An example implantable sensing system contains a sensing device that is inserted into a vein, an artery, or any other part of a human body where it could sense a desired parameter of the implant environment. An enzyme may be placed inside of the sensing device and employed for sensing. If the device is a glucose-sensing device, then a combination of glucose oxidase (GOx) and human serum albumin (HSA) may be utilized to form a sensor protein. During operation in a sensing device, glucose oxidase reacts with oxygen and oxidizes. The oxidation of glucose oxidase results in the formation of a hydroperoxy adduct, which transforms into hydrogen peroxide.

An obstacle to creating sensors that are long-lived and stable over time has been that glucose oxidase, when immobilized (e.g., for use in a sensor), undergoes oxidative inactivation by the aforementioned hydrogen peroxide over time. Since the lifetime of glucose sensors primarily depends on the lifetime of glucose oxidase, the effects of the peroxide on the glucose oxidase can severely limit the lifetimes of glucose sensors.

Prior processes for addressing the peroxide degradation of glucose oxidase have involved the use of additives or neutralizing agents for deactivating, removing or neutralizing peroxide. (Examples of such prior art are discussed below with respect to the Valdes et al. reference, the Stemmer patent, the Hatzinikolaou et al. article, the Wagner et al. patent, and the Aldrich Catalog page relating to Leuco-crystal violet) Embodiments of the present invention relate to a drastic change in direction of the state of the art by employing directed evolution techniques to formulate a glucose oxidase gene having desired peroxide resistant properties.

Evolution under non-stress circumstances takes years. Accordingly, evolution may be manipulated in embodiments of the invention for specific enzymatic functions. In embodiments of the invention, a technique known as directed evolution is employed to evolve glucose oxidase, to formulate a glucose oxidase that possesses improved resistance to peroxide. A glucose oxidase formulated pursuant to embodiments of the present invention may improve the longevity of a biosensor in which it is employed.

According to the claims under appeal, a method comprises creating a library of mutated glucose oxidase genes. Mutations of glucose oxidase genes may be obtained by performing polymerase chain reaction techniques, error-prone polymerase chain reaction techniques or gene shuffling techniques. Each of the library of mutants is inserted into a separate expression vector. Each expression vector is inserted into a host organism where a colony can grow, thereby replicating the mutated genes.

The library of colonies is then screened for desirable peroxide resistant properties. The colonies are screened by determining whether the colonies contain active glucose oxidase and determining whether the colonies have desired peroxide resistant properties. Determining whether the colonies have desired peroxide resistant properties involves incubating the colonies in peroxide and determining whether the colonies have active glucose oxidase after incubating, including measuring a concentration of the glucose oxidase.

In one embodiment, after the screening procedure, the glucose oxidase from one or more of the screened colonies may be mutated into a second generation library of mutants. The process may then proceed again with the second generation mutations. In other embodiments, this same process may be repeated many times on subsequent generations of mutated genes until a gene is formulated with suitable properties. In one embodiment the process is repeated from two to six times. In this manner, the mutations may be refined further to provide the desired peroxide resistant properties.

Those colonies that still contain active glucose oxidase after one or more mutation and incubation procedures may possess desirable peroxide resistant qualities. Glucose oxidase from those colonies still containing active glucose oxidase may be tested for functionality, for

example, by immobilizing the glucose oxidase in a sensor. In other embodiments of the invention, following at least a portion of the screening procedure, the environments of the colonies may be altered another time if desired, for example, by adding more peroxide.

The method recited in the pending claims of the present application can provide significant advantages over the prior art of record. The ability to form a stable enzyme which is peroxide resistant and which may be employed in an altered environment (oxygen free environment), such as a biosensor, can provide significant advantages in extending the life of biosensors. When used in an implanted medical device (such as an implanted blood glucose sensor), peroxide resistance and, thus, a capability for extending the life of the enzyme can provide considerable patient comfort and safety advances, for example, by reducing the frequency of surgical sensor replacements. Moreover, the ability to form enzymes with peroxide resistant properties suitable for biosensor applications in a relatively inexpensive, non-complicated and reliable process can provide significant advantages with respect to the ability to manufacture readily available supplies of the enzyme and, thus, increasing the availability of longer-life biosensors to more patients.

By a method in accordance with embodiments of the present invention, a glucose oxidase enzyme may be formulated to exhibit desired peroxide resistant properties. As such, further additives or other mechanisms for deactivating, removing or neutralizing peroxide may not be required. Thus, the disclosed method involves a distinct departure from the conventional direction of those skilled in the art.

VI. Grounds Of Rejection To Be Reviewed On Appeal

Claims 1, 3-8, 10-24 and 44-46 are rejected, as follows:

1. Claims 1, 3-8, 10-24 and 44-47 (*sic*, apparently 44-46) are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.
2. Claims 1, 3-8, 10-24 and 44-46 are rejected under 35 U.S.C. 103(a) as being unpatentable over Valdes et al., Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog.

As noted in Section III, above, the present appeal relates to each of the above rejections and, thus, all of the rejected claims (i.e., claims 1, 3-8, 10-24 and 44-46).

VII. Argument

1. Appeal Of Rejection Of Claims 1, 3-8, 10-24 and 44-47 Under 35 U.S.C. 112, Second Paragraph

Claims 1, 3-8, 10-24 and 44-47 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. This rejection is respectfully traversed. (With respect to previously-cancelled claim 47, this rejection is moot.) Applicant requests that the rejection be reversed and the rejected claims allowed in view of the following remarks.

In explaining the rejection, the Examiner stated:

“Claim 1 recites the phrase ‘determining whether the colonies contain active glucose oxidase comprises: detecting a concentration of active glucose oxidase’. It is not clear to the Examiner how the concentration of only active glucose oxidase is detected. Further, it is not clear how a concentration of glucose oxidase is detected from the colonies unless the glucose oxidase is first isolated. Therefore, the claim lacks essential steps: isolation step and detecting concentration of only active glucose oxidase.”

This rejection is respectfully traversed. In particular, it is submitted that one of ordinary skill in the art would understand the cited phrase (“detecting a concentration of active glucose oxidase”) in the context of the claimed invention. The original patent specification describes several manners in which a concentration of active glucose oxidase can be detected. For example, the original patent specification states:

“In one embodiment, the test for whether active glucose oxidase is present in a given colony comprises an assay which tests the production of peroxide. Peroxide is generated upon glucose oxidase reaction with glucose. In one embodiment, leuco-crystal-violet, a substrate that changes color in the presence of active peroxide, is employed. However, in other embodiments, other substances may also be used such as, but not limited to, aminoantipyrene, and the like.

In other embodiments, other methods can be used to test for the presence of active glucose oxidase. For example, the presence or absence of active glucose oxidase may be ascertainable by checking for fluorescence. The more fluorescent a given colony is, the more likely it is that it contains active glucose oxidase. Those skilled in the art will appreciate that further methods to test for the presence of glucose oxidase can be employed in other embodiments without deviating from the scope or spirit of the invention.” (page 10, line 16 through page 11, line 6).

As described in the above-quoted section of the original patent application, various methods may be used to detect active glucose oxidase, including using color changing materials (that change color in the presence of peroxide, which occurs when a concentration of active glucose oxidase is present) or checking for fluorescence (which occurs when a concentration of active glucose oxidase is present). One skilled in the art would understand that detection mechanisms function upon the presence of a suitable concentration of active glucose oxidase. The detection of active glucose oxidase necessarily involves a detection of a concentration of active glucose oxidase. One skilled in the art would be able to ascertain (or detect) whether or not a concentration of active glucose oxidase exists in a colony, based on the disclosure in the present application and conventional knowledge.

Furthermore, in applying the rejection under 35 U.S.C. 103 (discussed below), on page 6, lines 1-5 of the latest Office Action, the Examiner acknowledged that Hatzinikolaou et al. discloses a method of measuring glucose oxidase activity and concentration of glucose oxidase (citing pages 372-373). Thus, from ordinary knowledge in the art and the disclosures in the present application (quoted above) of various manners of testing for the presence of glucose oxidase one skilled in the art would know how to detect a concentration of glucose oxidase activity.

Also, the Examiner stated that it is not clear how the concentrations of the glucose oxidase is detected from the colonies unless the glucose oxidase is first isolated. The Examiner further stated that the claim lacks essential steps of isolation and assaying of the glucose oxidase. This characterization of the claimed subject matter is respectfully traversed. The method of claim 1 need not involve isolation of the glucose oxidase. The method may be carried out in situ

or upon isolation. Examples of manners of detecting a concentration of active glucose oxidase described in the above-quoted portion of the present specification may be carried out without requiring an isolation step. Thus, the claim need not be amended to require isolation. Accordingly, it is submitted that claim 1 is clear and definite to one of ordinary skill in the art.

In view of the forgoing, it is respectfully submitted that pending claims 1, 3-8, 10-24 and 44-46 are in compliance with 35 U.S.C. 112, second paragraph. The rejections of those claims is, therefore, respectfully traversed.

**2. Appeal Of Rejection Of Claims 1, 3-8, 10-24 and 44-46 Under 35 U.S.C.
103(a)**

Claims 1, 3-8, 10-24 and 44-46 are rejected under 35 U.S.C. 103(a) as being unpatentable over Valdes et al., Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog. This rejection is respectfully traversed. Applicant requests that the rejection be reversed and the rejected claims allowed over Valdes et al., Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog, in view of the following remarks.

Claim 1 recites a method for formulating an enzyme that is not disclosed by either Valdes et al. or Stemmer or Hatzinikolaou et al. or Wagner et al. or Aldrich Catalog. For example, the method of claim 1 recites, among other features:

“creating a library of mutated glucose oxidase genes,”

“growing colonies of the host organisms,” and

“screening the colonies for predefined, desired properties by determining whether the colonies contain active glucose oxidase and determining whether the colonies have predefined, desired peroxide resistant properties, wherein determining whether the colonies have predefined, desired peroxide resistant properties comprises: incubating the colonies in peroxide; and determining whether the colonies have active glucose oxidase after incubating the colonies in peroxide and, wherein determining whether the colonies contain active glucose oxidase comprises: detecting a concentration of active glucose oxidase.

The method recited in claim 1 recites several actions that, together, form the claimed method, where not any one of the above-cited references describes the combination of actions

recited in claim 1. In forming the rejection, the Examiner has selected and chosen certain pieces of various processes from each of the above-cited references and formed a mosaic of those pieces in an attempt to arrive at the present invention. The cited references provide no suggestion or motivation for the selecting pieces of the disclosed processes and combine them in the fashion that the Examiner suggests. Instead, the references, themselves, as well as other references of record teach a direction away from the present invention.

The mass of evidence of record in the application suggests that those skilled in the art were taking a direction that was completely different from that of the claimed invention. While the Examiner raises arguments as to obviousness to combine various parts of the cited references, none of the evidence of record supports the Examiner's proposal to select and combination portions of the various references. To the contrary, a number of references of record (including the primary reference relied upon by the Examiner) teach a direction different than the claimed invention and would lead one of ordinary skill in the art away from the claimed invention. Without the present disclosure as a guide, one of ordinary skill in the art would not have found it obvious to combine the above-cited references as suggested by the Examiner.

As described in more detail below:

- a. The prior art of record does not teach or suggest the claimed invention;
- b. None of the prior art of record provides any teaching or suggestion for the combination of the Valdes et al, Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog, as proposed by the Examiner, and the mass of evidence of record shows that the prior art teaches away from the claimed invention;
- c. Each of dependent claims 3-5, 10-18 and 20-24 recite further features that distinguish those claims from the prior art. Accordingly, those claims do not stand or fall with claim 1.

a. The Rejection Is Improper Because The Prior Art Does Not Teach Or Suggest The Claimed Invention.

In particular, neither Valdes et al. nor Stemmer nor Hatzinikolaou et al. nor Aldrich Catalog describe formulating a glucose oxidase enzyme by mutating glucose oxidases to make

them resistant to peroxide degradation. Moreover, one of ordinary skill in the art would not have been led by the prior art of record to mutate glucose oxidase genes, much less to mutate such genes and screen for desired peroxide resistance properties. Such procedures would have been a drastic departure from the state of the art and, without the benefit of the present specification as a guide, would not have been obvious to one of ordinary skill in the art.

The Examiner argues that Valdes et al. teaches that glucose oxidases in glucose sensors degrade over time due to hydrogen peroxide. The Examiner acknowledges that Valdes et al. do not teach a method of producing mutant glucose oxidase that is resistant to degradation from peroxide. (Final Office Action of November 1, 2006, pg. 5, ll. 3-6.) As discussed in more detail below, instead, Valdes et al. teach to address peroxide degradation by adding a chemical catalase or by attaching an immobilized enzyme to a support that deactivates hydrogen peroxide.

The Examiner refers to Valdes et al.'s statement that "[b]etter options" than replacing a degraded glucose oxidase sensor enzyme with a fresh enzyme is "to simply prevent the degradation of the enzyme" (Final Office Action of November 1, 2006, pg. 4, ll. 15-18, citing Valdes et al., pg. 375, ll. 2-5). The Examiner attempts to use that statement out of context, as a springboard to imply that Valdes et al. would have suggested a process involving creating mutated genes of glucose oxidase and screening colonies in the manner recited in the present claims. However, Valdes et al. immediately follow that statement with a description of the use of chemical additives as the so-called "better options." Accordingly, Valdes et al. teach a specific direction (use of chemical additives) that departs from the then-conventional process of replacing a degraded enzyme with a fresh enzyme.

Valdes et al. is not the only reference of record that teaches that the direction taken by those skilled in the art was to use chemical additives. Indeed, other references of record similarly teach that direction of the art (e.g., U.S. Patent No. 6,689,265 to Heller et al. and the article titled "Glucose ENFET doped with MnO₂ powder" by Yin et al, attached as Exhibits 1 and 2, respectively). Neither Valdes et al., nor any prior art of record that relates to peroxide degradation of glucose oxidase, describe or suggest the use of a gene mutation method for addressing peroxide degradation of glucose oxidase. Instead, as described in more detail below, Valdes et

al. and other references of record show that the direction taken by those skilled in the art was away from the method of the presently claimed invention.

Because of this lack of disclosure in Valdes et al., the Examiner attempts to select pieces of each of the Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog. However, none of those references teach formulating a glucose oxidase enzyme by mutating glucose oxidases to make them resistant to peroxide degradation.

For example, as noted above, claim 1 recites a method for formulating an enzyme that includes, among other features, “obtaining a library of glucose oxidase genes” and “creating a library of mutated glucose oxidase genes.” The Examiner stated that Stemmer discloses a method of producing mutant enzymes by obtaining a library of genes of interest and creating a library of mutated genes by multiple cycles. (Final Office Action dated November 1, 2006, pg. 5, ll. 10-12.)

However, Stemmer provides no teaching or suggestion of obtaining a library of glucose oxidase genes or a library of mutated glucose oxidase genes. Stemmer describes common DNA mutation techniques, some of which are noted in the present application (e.g., at page 8, ll. 21-23). Indeed, even the present application refers to common ways to create a library of mutant genes, including Error-Prone Polymerase Chain Reaction (“Error-Prone PCR”), gene shuffling and other processes. However, it is well beyond the teaching of Stemmer or those common mutation processes to use such techniques to form a library of glucose oxidase genes or to create a library of mutated glucose oxidase genes and then to screen colonies for desired peroxide resistant properties in accordance with the presently claimed method. Stemmer’s description of mutating DNA for the production of nucleic acid fragments or polynucleotides encoding mutant proteins, provides no suggestion or motivation to create a library of mutated glucose oxidase genes.

The Examiner stated that Hatzinikolaou et al. discloses a library of glucose oxidase genes known in the art, a method of isolating and purifying glucose oxidase and methods of measuring glucose oxidase activity and concentration of glucose oxidase. However, Hatzinikolaou et al. describe isolating and characterizing a new synthesized glucose oxidase for purposes of

conducting certain specified analyses (described on pages 373 and 374 of the Hatzinikolaou et al. reference), none of which relate to resistance to hydrogen peroxide.

While gene libraries have been employed by those skilled in the art for gene analysis, Hatzinikolaou et al. provide no suggestion to use such libraries in the formulation of an enzyme by directed evolution. Hatzinikolaou et al. teaching of using gene libraries to analyze characteristics of a gene provides no motivation or suggestion to do anything more than to analyze the specific new synthesized glucose oxidase for the specific characteristics described on pages 373 and 374 of that reference. The Examiner has picked only the feature of forming a gene library of glucose oxidase gene from Hatzinikolaou et al.'s overall process and seeks to combine that teaching with Stemmer (and other references cited in the rejection).

However, Hatzinikolaou et al.'s purpose of forming a library of a particular new simulated glucose oxidase (for analyzing the characteristics of the new simulated glucose oxidase described in that reference) would have no applicable purpose in any mutation process described by Stemmer. Once Hatzinikolaou et al. obtains and isolates a sample of the new glucose oxidase, Hatzinikolaou et al. conducts analysis on the isolated sample. Mutating the sample according to Stemmer would not allow Hatzinikolaou et al. to analyze the characteristics of the simulated glucose oxidase (as the mutations could effect the detection of characteristics under analysis). Accordingly, it would not have been obvious to look to Hatzinikolaou et al. as a teaching of obtaining a library of glucose oxidase genes that are to be mutated to create a library of mutated glucose oxidase genes. The Examiner's suggestion to combine Hatzinikolaou et al. with Stemmer is, therefore, respectfully traversed. Moreover, Hatzinikolaou et al. does not disclose or suggest screening colonies for active glucose oxidase predefined, desired peroxide resistant properties.

Claim 1 of the present application further recites: "screening the colonies for predefined, desired properties by determining whether the colonies contain active glucose oxidase and determining whether the colonies have predefined, desired peroxide resistant properties." Claim 1 further recites that "determining whether the colonies have predefined, desired peroxide resistant properties comprises: incubating the colonies in peroxide; and determining whether the colonies have active glucose oxidase after incubating the colonies in peroxide, wherein

determining whether the colonies contain active glucose oxidase comprises: detecting a concentration of active glucose oxidase.”

Because Stemmer does not relate to obtaining a library of glucose oxidase genes or a library of mutated glucose oxidase genes, it follows that Stemmer also does not describe screening colonies by determining whether the colonies contain active glucose oxidase and determining whether the colonies have predefined, desired peroxide resistant properties. Also, Hatzinikolaou et al. provide no teaching or suggestion of screening colonies for active glucose oxidase having a desired peroxide resistant property. While the combination of those references is traversed for reasons noted above, no combination of those references could lead to screening colonies for active glucose oxidase having a desired peroxide resistant property because neither of those references, alone, describe such a feature. Moreover, as noted above, because Valdes et al. also do not teach to screening colonies for active glucose oxidase having a desired peroxide resistant property and, instead, teach a very different direction (addition of chemicals to reduce peroxide degradation), the Examiner’s suggestion to combine Valdes et al. with Stemmer and Hatzinikolaou et al. is traversed and still would not lead to the present invention.

The Examiner cited the Wagner reference for disclosing a method of determining glucose oxidase activity in a sensor by measuring fluorescence. Wagner’s method is used for continuous monitoring of glucose in a body fluid. Wagner neither describes nor suggests formulating an enzyme, much less creating a library of mutated glucose oxidase genes, screening colonies for predefined, desired properties by determining whether the colonies contain active glucose oxidase or determining whether the colonies have predefined desired peroxide resistant properties. Accordingly, Wagner does not address the above-noted distinctions between the claimed invention and the Valdes et al., Stemmer and Hatzinikolaou et al. references. Thus, the combination of Wagner with those other references (as suggested by the Examiner) could not result in the claimed invention.

The Examiner cited the Aldrich Catalog as describing Leuco-crystal violet dyes as common fluorescent dyes. The cited portion of the Aldrich Catalog neither describes nor suggests formulating an enzyme, much less creating a library of mutated glucose oxidase genes, screening colonies for predefined, desired properties by determining whether the colonies contain

active glucose oxidase or determining whether the colonies have predefined desired peroxide resistant properties. Accordingly, the cited portion of the Aldrich Catalog does not address the above-noted distinctions between the claimed invention and the Valdes et al., Stemmer, Hatzinikolaou et al. and Wagner references. Thus, the combination of the cited portion of the Aldrich Catalog with those other references (as suggested by the Examiner) could not result in the claimed invention.

None of the cited references describes or suggests creating a library of mutated glucose oxidase genes and then screening colonies for active glucose oxidase having a desired peroxide resistant property. Accordingly, the combination of the references (as suggested by the Examiner) could not result in the claimed invention. The rejection of claims 1, 3-8, 10-24 and 44-46 under 35 U.S.C. 103(a) is, therefore, respectfully traversed and should be reversed.

b. The Rejection Is Improper Because Prior Art Provides No Motivation To Combine And Teaches Away From The Combination Suggested By The Examiner.

Because of the above-noted lack of disclosure in Valdes et al. (of creating a library of mutated glucose oxidase genes and screening colonies for active glucose oxidase predefined, desired peroxide resistant properties), the Examiner attempts to select pieces of each of the Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog and combine those with the Valdes et al. reference. The present rejection is the second attempt by the Examiner to combine the Valdes et al. reference with other references.

An attempt to combine the Valdes et al. reference with other references of record (i.e., article titled "Current Protocols in Molecular Biology") was previously made in the rejections raised in the Final Office Action of July 12, 2005. In Applicant's Appeal Brief filed on December 7, 2005, Applicant explained that the combination was improper because of the direction of teaching of Valdes et al. (of using chemical additives) would have led one of ordinary skill in the art away from gene mutation methods taught by the article titled "Current Protocols in Molecular Biology." In response to Applicant's Appeal Brief of December 7, 2005, the Examiner re-opened prosecution and apparently withdrew previous rejections over prior art (as those previous rejections were not applied), but raised a new ground of rejection by

attempting to combine the Valdes et al. reference with Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog.

None of the Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog references provide any teaching of creating a library of mutated glucose oxidase genes and screening colonies for active glucose oxidase with predefined, desired peroxide resistant properties, or methods of formulating a glucose oxidase enzyme with peroxide resistance. Accordingly, each of those references is no more relevant to those features as was the article titled "Current Protocols in Molecular Biology". No explanation has been provided as to why the Examiner found it appropriate to not re-assert (e.g., withdraw) the rejections based on the attempt to combine Valdes et al. with the article titled "Current Protocols in Molecular Biology" and then applying a new rejection based on an attempt to combine Valdes et al. with the Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog references. At least some of the same reasons that applied to the traversal of the rejection of the claims over Valdes et al. in view of the article titled "Current Protocols in Molecular Biology" (and that resulted in the Examiner's apparent withdrawal of the rejection after Applicant filed the Appeal Brief of December 7, 2005) would apply to the present rejection based on the attempt to combine Valdes et al. with the Stemmer, Hatzinikolaou et al., Wagner et al. and Aldrich Catalog references.

In addition to the inconsistent issuance of a new rejection based on an attempt to combine Valdes et al. with other references relating to gene mutation (after the combination of Valdes et al. with the article titled "Current Protocols in Molecular Biology" was traversed and apparently overcome), the rejection under 35 U.S.C. 103 is improper because of a lack of motivation (without the present disclosure as a guide) to pick and choose various discrete features out of multiple references and combine those features as suggested by the Examiner.

As noted above, the Examiner had acknowledged that "Valdes et al. does not teach a method of producing mutant glucose oxidase that is resistant to degradation from peroxide and detecting concentration of active glucose oxidase in sensors and using fluorescence." (Final Office Action dated November 1, 2006, pg. 5, ll. 3-6.) Accordingly, the Examiner argues it would have been obvious to combine Stemmer, Hatzinikolaou et al., Wagner et al. and the Aldrich Catalog references with the Valdes process.

However, the Examiner cites no teaching in either the Valdes et al. reference or the Stemmer, Hatzinikolaou et al., Wagner et al. or Aldrich Catalog references (or any other prior art) for incubating mutated colonies of glucose oxidase with hydrogen peroxide. In fact, neither Valdes et al., Stemmer, Hatzinikolaou et al., Wagner et al. nor the Aldrich Catalog references provide any motivation or suggestion for creating a library of mutated glucose oxidase genes and screening colonies for active glucose oxidase and desired peroxide resistant properties. Indeed, Valdes et al. teach away from such methods by, instead, referring to conventional procedures (using additives for deactivating or destroying hydrogen peroxide and, thus, teach away from such a method, as follows:

“To prohibit the H_2O_2 from degrading the GOD enzyme, it has been proposed that catalase be coimmobilized with GOD ... The addition of catalase in either the GOD itself, or to the incubating solution has resulted in a slower deactivation of the GOD enzyme ... A long term remedy of the degradation of GOD by H_2O_2 could be the immobilization and attachment of the enzyme to a support that deactivates H_2O_2 , as it is being produced. Such as study was conducted by Cho², using the peroxide decomposition catalyst, activated carbon. In a study conducted by Carter¹⁹, the best results were obtained with activated carbon, impregnated with ruthenium. This combination was able to destroy hydrogen peroxide and stabilized the enzyme.” (Valdes et al., pg. 375, col. 1, l.18 to col. 2, l. 6.)

Not only does Valdes et al. fail to teach or suggest to mutate glucose oxidase and screen mutated glucose oxidase for peroxide resistance properties, but, in the above-quoted statement, Valdes et al. further teaches to use other, very different procedures (conventional in the art) to address degradation effects of peroxide on glucose oxidase. Thus, the Valdes et al. reference shows that the direction taken by those most skilled in the art involved employing materials, additives, or the like that deactivate peroxide.

Additional prior art of record also describes conventional “additive” processes for removing or neutralizing peroxide such as by adding an antioxidant or peroxidase to the glucose oxidase to break down peroxide or by coating the glucose oxidase enzyme with a protective coating, including U.S. Patent No. 6,689,265 to Heller et al. (Exhibit 1) and the article titled “Glucose ENFET doped with MnO_2 powder” by Yin et al (Exhibit 2). Those prior art references further emphasize that the direction taken by those skilled in the art for addressing the peroxide degradation of glucose oxidase is wholly different from the direction of the present invention. In

U.S. Patent No. 6,689,265 to Heller et al., a peroxide generating enzyme may include a sufficiently thick, natural, electrically insulating protein or glycoprotein layer. (See column 6, lines 59-67 of the Heller et al. patent, Exhibit 1.) Heller et al. also disclose an alternative embodiment in which a peroxide generating enzyme is immobilized in a non-conducting inorganic or organic polymeric matrix. (See column 7, lines 3-11 of the Heller et al. patent, Exhibit 1.) Also, Heller et al. describe a first layer enzyme 11 (peroxidase) that reduces peroxide generated from a second layer (glucose oxidase layer) 13. The Yin et al. article describes the addition of MnO_2 to catalyze peroxide and produce water and oxygen therefrom. (Yin, Exhibit 2, Abstract and pg. 188, col. 1, ll. 20-34.)

Thus, both the Heller et al. patent and the Yin et al. article show that the direction taken by those skilled in the art is to provide additives or complex multi-layer sensor structures to remove hydrogen peroxide. These references, in addition to Valdes et al.'s express references to conventional uses of additives, show that those skilled in the art were not considering mutating glucose oxidase genes and growing and screening colonies for peroxide resistance, but instead were attempting to address the peroxide production issue by removing or neutralizing peroxide with additives (not by altering the glucose oxidase). The state and direction of the prior art, as evidenced by Valdes et al., Heller et al. and Yin et al., was a wholly different direction than that taken by the present Applicants (including creating a library of mutated glucose oxidase enzyme genes and screening colonies for desirable properties by determining whether the colonies contain active glucose oxidase and determining whether the colonies have desired peroxide resistant properties.) Accordingly, the mass of evidence of record (including the primary reference relied upon by the Examiner) teaches one skilled in the art in a direction different from (and away from) the present invention.

Without the present disclosure as a guide, one of ordinary skill in the art would not have found Valdes et al.'s discussion of the degradation of glucose oxidase as a prompt or suggestion to employ a DNA mutation process as described Stemmer. Instead, as noted above, one of ordinary skill in the art would have looked to conventional manners of removing peroxide, such as additives for removing or neutralizing peroxide. Accordingly, the rejection of 1, 3-8, 10-24 and 44-46 under 35 U.S.C. 103(a) is further respectfully traversed.

The fact that the primary reference (Valdes et al.) teach away from the claimed invention and the combination suggested by the Examiner, shows that a *prima facie* case of obviousness has not been raised. Numerous Federal Circuit decisions recognize that an invention will not be deemed obvious in a patent law sense when one or more prior art references “teach away” from the invention. For example, the Federal Circuit stated “as a useful general rule, that references that teach away cannot serve to create a *prima facie* case of obviousness.” *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1354, 60 USPQ2d 1001 (Fed. Cir. 2001).

Furthermore, “an applicant may rebut a *prima facie* case of obviousness by showing that the prior art teaches away from the claimed invention in any material respect.” *In re Peterson*, 315 F.3d 1325, 1331, 65 USPQ2d 1379 (Fed. Cir. 2003). Also see, *Gillette Co. v. S.C. Johnson & Sons, Inc.*, 919 F.2d 720, 724, 16 USPQ2d 1923, 1927 (Fed. Cir. 1990)(the closest prior art reference “would likely discourage the art worker from attempting the substitution suggested by [the inventor/patentee]”) and *Singh v. Brake*, 317 F.3d 1334, 1346, 65 USPQ2d 1641 (Fed. Cir. 2003)(“whether or not a reference ‘teaches away’ from a claimed invention” is “relevant in determining whether or not a claimed invention would have been obvious”).

When patentability turns on the question of obviousness, the search for and analysis of the prior art includes evidence relevant to the finding of whether there is a teaching, motivation, or suggestion to select and combine the references relied on as evidence of obviousness. (underline added for emphasis.) See, e.g., *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1351—52, 60 USPQ2d 1001, 1008 (Fed. Cir. 2001) (“the central question is whether there is reason to combine [the] references,” a question of fact drawing on the *Graham* factors).

Conclusory statements that prior art references provide motivation to combine, or statements of motivation derived from the Applicant’s own specification, are not sufficient to set forth a *prima facie* case of obviousness. “The factual inquiry whether to combine references must be thorough and searching.” *Id.* It must be based on objective evidence of record. This precedent has been reinforced in myriad decisions. See, e.g., *Brown & Williamson Tobacco Corp. v. Philip Morris Inc.*, 229 F.3d 1120, 1124—25, 56 USPQ2d 1456, 1459 (Fed. Cir. 2000) (“a showing of a suggestion, teaching, or motivation to combine the prior art references is an ‘essential component of an obviousness holding’”) (quoting *C.R. Bard, Inc., v. M3 Systems, Inc.*,

157 F.3d 1340, 1352, 48 USPQ2d 1225, 1232 (Fed. Cir. 1998)); *In re Dembiczak*, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999) (“Our case law makes clear that the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references.”); *In re Dance*, 160 F.3d 1339, 1343, 48 USPQ2d 1635, 1637 (Fed. Cir. 1998) (there must be some motivation, suggestion, or teaching of the desirability of making the specific combination that was made by the applicant); *In re Fine*, 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988) (“teachings of references can be combined *only* if there is some suggestion or incentive to do so.”) (emphasis in original) (quoting *ACS Hosp. Sys., Inc. v. Montefiore Hosp.*, 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984)).

As noted above, the Examiner has not shown any motivation or suggestion in the prior art that would have led one skilled in the art to select a mutation and screening process described by the Stemmer for creating a library of mutated glucose oxidase and screening colonies for peroxide resistant properties. In fact, Valdes et al and other prior art of record show that a selection of a mutation and screening process would have been a drastic diversion from the direction taken by those most skilled in the prior art.

The legal authority expresses the requirement for a showing of specificity in the prior art of motivation to select components to combine. See, e.g., *In re Kotzab*, 217 F.3d 1365, 1371, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000) (“particular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed”); *In re Rouffet*, 149 F.3d 1350, 1359, 47 USPQ2d 1453, 1459 (Fed. Cir. 1998) (“even when the level of skill in the art is high, the Board must identify specifically the principle, known to one of ordinary skill, that suggests the claimed combination. In other words, the Board must explain the reasons one of ordinary skill in the art would have been motivated to select the references and to combine them to render the claimed invention obvious.”); *In re Fritch*, 972 F.2d 1260, 1265, 23 USPQ2d 1780, 1783 (Fed. Cir. 1992) (the examiner can satisfy the burden of showing obviousness of the combination “only by showing some objective teaching in the prior art or that knowledge generally available to one of

ordinary skill in the art would lead that individual to combine the relevant teachings of the references”).

While the Valdes et al., Stemmer and Hatzinikolaou et al. references, themselves, provide no motivation or suggestion, the Examiner argues that one of ordinary skill in the art would have been motivated to do so in order to generate active glucose oxidase that is resistant to peroxide. (Final Office Action dated November 1, 2006, pg. 7, ll. 4-7.) While this is the likely conclusion, after reading the present disclosure as a guide, the references of record actually teach to do something very different (add chemicals) to reduce peroxide degradation of glucose oxidase. Thus, the mass of evidence of record shows that the motivation provided by the cited references would have been to reduce peroxide degradation by adding chemicals as taught by Valdes et al., U.S. Patent No. 6,689,265 to Heller et al. (Exhibit 1) and the article titled “Glucose ENFET doped with MnO₂ powder” by Yin et al (Exhibit 2).

In addition, the Examiner argues that one of ordinary skill in the art would have had a “reasonable expectation of success.” However, without the present disclosure as a guide, one of ordinary skill in the art would not have selected gene mutation processes and glucose oxidase purifying, isolating and measuring processes to modify Valdes et al.’s disclosed solution to peroxide degradation of glucose oxidase. Valdes et al. teaches solutions to the peroxide degradation problem (by using chemical additives) and would have led one skilled in the art in the direction of those solutions. Stemmer does not appear to mention glucose oxidase anywhere in its disclosure. Hatzinikolaou et al. fails to provide any motivation or suggest any relation to a gene mutation procedure or of addressing peroxide degradation of glucose oxidase. Moreover, the whole purpose of Hatzinikolaou et al. (to analyze a specific new simulated glucose oxidase) is not consistent with Stemmer’s method of DNA reassembly or production of nucleic acid fragments or polynucleotides encoding mutant proteins.

The Examiner’s conclusory statements of suggestion to combine and the Examiner’s argument of “reasonable expectation of success” fail to address the significant issue of why one skilled in the art would have been motivated to select a process as described by Stemmer, to change the direction taken by those most skilled in the prior art as described by Valdes et al. The Examiner’s argument that a “reasonable expectation of success” would have motivated the

combination, is contrary to the express teachings of the prior art. The prior art teaches that those most skilled in the art were taking a wholly different direction to address peroxide degradation of glucose oxidase and, thus, would have found it unreasonable (not reasonable) to change the course of direction from that of the state of the art.

More specifically, Valdes et al. refer to completely different directions taken by those most skilled in the art, whereby the glucose oxidase enzyme is immobilized and attached to a support that deactivates peroxide. "A reference may be said to teach away when a person of ordinary skill, upon reading the reference, ... would be led in a direction divergent from the path that was taken by the applicant." *Tec Air, Inc. v. Denso Mfg. Mich. Inc.*, 192 F.3d 1353, 1360, 52 USPQ2d 1294, 1298 (Fed. Cir. 1999). Valdes et al., directly refers the reader to conventional methods of addressing peroxide degradation of glucose oxidase that employ additives for destroying or neutralizing peroxide (which is quite different from creating a library of mutated genes and screening for desired peroxide resistant properties).

Because the Examiner has not shown any motivation or suggestion in the prior art that would have led one skilled in the art to select Stemmer's mutation process with the Valdes et al. reference, the Examiner has not raised a *prima facie* case of obviousness. Therefore, the rejection of 1, 3-5, 12-24 and 44-45 under 35 U.S.C. 103(a) is respectfully traversed.

The Wagner reference does not address the above-noted distinctions between the claims and the Valdes et al., Stemmer and Hatzinikolaou references. Indeed, the Wagner reference was cited, according to the Examiner, for disclosing a method of determining glucose oxidase activity via a sensor by measuring fluorescence emission from a dye, wherein oxidation of glucose by active glucose oxidase reduces the fluorescence emission. However, Wagner does not teach or suggest formulating a glucose oxidase enzyme by mutating glucose oxidases to make them resistant to peroxide degradation. Accordingly, the combination of Wagner with the above-discussed references (the Valdes et al., Stemmer and Hatzinikolaou references) would not lead to the presently claimed invention. The Aldrich Catalog also does not address the above-noted distinctions between the claims and the Valdes et al., Stemmer and Hatzinikolaou references.

c. Each of dependent claims 3-5, 10-18 and 20-24 recite further features that distinguish those claims from the prior art.

Each of dependent claims 3-5, 10-18, and 20-24 recite further features that distinguish those claims from the prior art. In particular, each of those claims recites features relating to colonies of mutated glucose oxidase gene. As described above, neither Valdes et al. nor the Stemmer, Hatzinikolaou et al, Wagner et al or Aldrich Catalog references, alone or in combination, describe or suggest mutating glucose oxidase and screening for active glucose oxidase and predefined, desired peroxide resistant properties. In that regard, those references also do not disclose or suggest the additional processing recited in dependent claims 3-5, 10-18, and 20-24, including:

1. "screening the colonies for desirable properties further comprises testing glucose oxidase from the colonies for functionality" (claim 3);
2. "determining whether the colonies have peroxide resistant properties is only performed if results of determining whether the colonies contain active glucose oxidase are positive" (claim 4);
3. "testing glucose oxidase from the colonies for functionality is only performed if results of determining whether the colonies contain active glucose oxidase are positive and if results of determining whether the colonies have peroxide resistant properties are positive" (claim 5);
4. "testing glucose oxidase from the colonies for functionality comprises employing glucose oxidase from the colonies in sensors" (claim 10);
5. "extracting glucose oxidase from the colonies; immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies; placing the immobilized glucose oxidase in a sensor; and testing the sensor" (claim 11);
6. "employing an ionic column to extract glucose oxidase from the colonies" (claim 12);
7. "removing the glucose oxidase from the colonies; purifying the glucose oxidase; and characterizing the glucose oxidase" (claim 13);

8. “grinding the colonies in a homogenizer into cell components” (claim 14);
9. “fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer” (claim 15);
10. “disrupting the colonies into cell components via sonication” (claim 16);
11. “fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication” (claim 17);
12. “purifying the glucose oxidase by employing chromatography methods” (claim 18).
13. “creating at least one mutated glucose oxidase gene comprises employing polymerase chain reaction techniques to create at least one mutated glucose oxidase gene” (claim 20);
14. “employing error-prone polymerase chain reaction techniques to create at least one mutated glucose oxidase gene” (claim 21);
15. “employing gene shuffling techniques to create at least one mutated glucose oxidase gene” (claim 22);
16. “creating a next generation of mutated glucose oxidase genes after screening the colonies for desirable properties” (claim 23); and
17. “creating a next generation of mutated glucose oxidase genes is repeated approximately 2 to 6 times” (claim 24).

The rejection of claims 3-5, 8, 10-18 and 20-24 is, therefore further respectfully traversed and should be reversed.

VIII. Conclusion

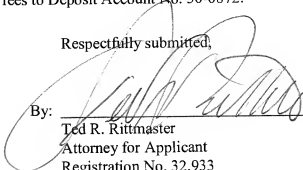
In view of the foregoing, it is respectfully submitted that claims 1, 3-8, 10-24 and 44-46 are in condition for allowance and the application should be allowed in its present form. In particular, it is respectfully submitted that the presently pending rejections of claims 1, 3-8, 10-24

and 44-46 are improper and should be reversed for reasons as discussed above. In that regard, each of claims 1, 3-8, 10-24 and 44-46 is in condition for allowance.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 50-0872. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 50-0872. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 50-0872.

Respectfully submitted,

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APPENDIX A

Claims Appendix

1. (Previously Presented) A method for formulating an enzyme comprising:
obtaining a library of glucose oxidase genes;
creating a library of mutated glucose oxidase genes;
introducing each mutated glucose oxidase gene of the library into separate expression
vectors;
inserting the expression vectors into non-human host organisms;
growing colonies of the host organisms;
screening the colonies for predefined, desired properties by determining whether the
colonies contain active glucose oxidase and determining whether the colonies have predefined,
desired peroxide resistant properties,
wherein determining whether the colonies have predefined, desired peroxide resistant
properties comprises:
incubating the colonies in peroxide; and
determining whether the colonies have active glucose oxidase after incubating the
colonies in peroxide,
wherein determining whether the colonies contain active glucose oxidase comprises:
detecting a concentration of active glucose oxidase.
2. Cancelled.
3. (Previously Presented) A method for formulating an enzyme according to
claim 1, wherein screening the colonies for desirable properties further comprises testing glucose
oxidase from the colonies for functionality to function as a sensor enzyme in a sensor.
4. (Original) A method for formulating an enzyme according to claim 1, wherein
determining whether the colonies have peroxide resistant properties is only performed if results
of determining whether the colonies contain active glucose oxidase are positive.
5. (Original) A method for formulating an enzyme according to claim 3, wherein
testing glucose oxidase from the colonies for functionality is only performed if results of
determining whether the colonies contain active glucose oxidase are positive and if results of
determining whether the colonies have peroxide resistant properties are positive.

6. (Previously presented) A method for formulating an enzyme according to claim 1, wherein determining whether the colonies have active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

7. (Original) A method for formulating an enzyme according to claim 6, wherein the substance is leuco-crystal-violet.

8. (Previously presented) A method for formulating an enzyme according to claim 1, wherein determining whether the colonies have active glucose oxidase comprises checking for fluorescence.

9. Cancelled.

10. (Previously presented) A method for formulating an enzyme according to claim 3, wherein testing glucose oxidase from the colonies for functionality comprises employing glucose oxidase from the colonies in sensors.

11. (Original) A method for formulating an enzyme according to claim 10, wherein employing glucose oxidase from the colonies in sensors comprises:
extracting glucose oxidase from the colonies;
immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies;
placing the immobilized glucose oxidase in a sensor; and
testing the sensor.

12. (Original) A method for formulating an enzyme according to claim 11, wherein extracting glucose oxidase from the colonies comprises employing an ionic column to extract glucose oxidase from the colonies.

13. (Original) A method for formulating an enzyme according to claim 11, wherein extracting glucose oxidase from the colonies comprises:
removing the glucose oxidase from the colonies;
purifying the glucose oxidase; and
characterizing the glucose oxidase.

14. (Original) A method for formulating an enzyme according to claim 13, wherein removing the glucose oxidase from the colonies comprises grinding the colonies in a homogenizer into cell components.

15. (Original) A method for formulating an enzyme according to claim 14, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer.

16. (Original) A method for formulating an enzyme according to claim 13, wherein removing the glucose oxidase from the colonies comprises disrupting the colonies into cell components via sonication.

17. (Original) A method for formulating an enzyme according to claim 16, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication.

18. (Original) A method for formulating an enzyme according to claim 13, wherein purifying the glucose oxidase comprises purifying the glucose oxidase by employing chromatography methods.

19. (Previously Presented) A method for formulating an enzyme according to claim 1, wherein the glucose oxidase is obtained from an organism and wherein the organism is selected from a group consisting of *Aspergillus Niger*, *Penicillium funiculosum*, *Saccharomyces cerevisiae*, and *Escherichia Coli*.

20. (Original) A method for formulating an enzyme according to claim 1, wherein creating at least one mutated glucose oxidase gene comprises employing polymerase chain reaction techniques to create at least one mutated glucose oxidase gene.

21. (Original) A method for formulating an enzyme according to claim 1, wherein creating at least one mutated glucose oxidase gene comprises employing error-prone polymerase chain reaction techniques to create at least one mutated glucose oxidase gene.

22. (Original) A method for formulating an enzyme according to claim 1, wherein creating at least one mutated glucose oxidase gene comprises employing gene shuffling techniques to create at least one mutated glucose oxidase gene.

23. (Original) A method for formulating an enzyme according to claim 1, wherein the method further comprises creating a next generation of mutated glucose oxidase genes after screening the colonies for desirable properties.

24. (Original) A method for formulating an enzyme according to claim 23, wherein creating a next generation of mutated glucose oxidase genes is repeated approximately 2 to 6 times.

25. (Withdrawn) An enzyme formulated according to the method of claim 1.

26. (Withdrawn) A method for formulating an enzyme comprising:
obtaining an organism with a glucose oxidase gene;
growing multiple colonies of the organism;
altering the environment of the colonies; and

screening the colonies to identify colonies with active glucose oxidase after altering the environment of the colonies.

27. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein the organism is selected from a group consisting of *Aspergillus Niger*, *Penicillium funiculosum*, *Saccharomyces cerevisiae*, and *Escherichia Coli*.

28. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein altering the environment of the colonies comprises introducing peroxide to the colonies.

29. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein screening the colonies to identify colonies with active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

30. (Withdrawn) A method for formulating an enzyme according to claim 29, wherein the substance is leuco-crystal-violet.

31. (Withdrawn) A method for formulating an enzyme according to claim 30, wherein screening the colonies to identify colonies with active glucose oxidase comprises checking for fluorescence.

32. (Withdrawn) A method for formulating an enzyme according to claim 26, wherein the method further comprises testing the colonies with active glucose oxidase for functionality after screening the colonies to identify colonies with active glucose oxidase.

33. (Withdrawn) A method for formulating an enzyme according to claim 32, wherein the method further comprises continuing to alter the environments of the colonies until the colonies with active glucose oxidase are of a suitable number to proceed with testing the colonies with active glucose oxidase for functionality.

34. (Withdrawn) A method for formulating an enzyme according to claim 32, wherein testing the colonies with active glucose oxidase for functionality comprises employing glucose oxidase from the colonies in sensors.

35. (Withdrawn) A method for formulating an enzyme according to claim 32, wherein testing the colonies with active glucose oxidase for functionality comprises:

- extracting glucose oxidase from the colonies;
- immobilizing the glucose oxidase after extracting the glucose oxidase from the colonies;
- placing the immobilized glucose oxidase in a sensor; and

testing the sensor.

36. (Withdrawn) A method for formulating an enzyme according to claim 35, wherein extracting glucose oxidase from the colonies comprises employing an ionic column to extract glucose oxidase from the colonies.

37. (Withdrawn) A method for formulating an enzyme according to claim 35, wherein extracting glucose oxidase from the colonies comprises:

- removing the glucose oxidase from the colonies;
- purifying the glucose oxidase; and

characterizing the glucose oxidase.

38. (Withdrawn) A method for formulating an enzyme according to claim 37, wherein removing the glucose oxidase from the colonies comprises grinding the colonies in a homogenizer into cell components.

39. (Withdrawn) A method for formulating an enzyme according to claim 38, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after grinding the colonies in a homogenizer.

40. (Withdrawn) A method for formulating an enzyme according to claim 37, wherein removing the glucose oxidase from the colonies comprises disrupting the colonies into cell components via sonication.

41. (Withdrawn) A method for formulating an enzyme according to claim 40, wherein removing the glucose oxidase from the colonies further comprises fractionating the cell components employing centrifugation and differential solubility after disrupting the colonies via sonication.

42. (Withdrawn) A method for formulating an enzyme according to claim 37, wherein purifying the glucose oxidase comprises purifying the glucose oxidase by employing chromatography methods.

43. (Withdrawn) An enzyme formulated according to the method of claim 26.

44. (Previously Presented) The method of formulating an enzyme according to claim 1, wherein the host organism comprises a microorganism.

45. (Original) The method of formulating an enzyme according to claim 1, wherein determining whether the colonies contain active glucose oxidase further comprises isolating the glucose oxidase.

46. (Previously Presented) The method of formulating an enzyme according to claim 1, wherein screening the colonies for desirable properties further comprises:
isolating the glucose oxidase;
placing the glucose oxidase in a sensor; and
testing the sensor.

47. (Cancelled)

48. (Withdrawn) A method for making a biosensor comprising:
obtaining a library of glucose oxidase genes;
creating a library of mutated glucose oxidase genes;
introducing each mutated glucose oxidase gene of the library into separate expression vectors;
inserting the expression vectors into a host;
growing colonies of the host;
screening the colonies for desirable properties by determining whether the colonies contain active glucose oxidase and determining whether the colonies have peroxide resistant properties; and
placing a glucose oxidase gene having desirable properties into a sensor,
wherein determining whether the colonies have peroxide resistant properties comprises:
incubating the colonies in peroxide; and
determining whether the colonies have active glucose oxidase after incubating the colonies in peroxide, and
wherein determining whether the colonies contain active glucose oxidase comprises:
measuring a concentration of the glucose oxidase.

49. (Withdrawn) A method for making a biosensor according to claim 47, wherein screening the colonies for desirable properties further comprises testing glucose oxidase from the colonies for functionality.

50. (Withdrawn) A method for making a biosensor according to claim 47, wherein determining whether the colonies have active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

51. (Withdrawn) A method for making a biosensor according to claim 47, wherein determining whether the colonies have active glucose oxidase comprises employing a substance that changes color in the presence of active glucose oxidase.

52. (Withdrawn) A method for making a biosensor according to claim 51, wherein the substance is leuco-crystal-violet.

53. (Withdrawn) A method for making a biosensor according to claim 47, wherein determining whether the colonies have active glucose oxidase comprises checking for fluorescence.

54. (Withdrawn) A method for making a biosensor according to claim 47, wherein the host is a host organism.

EXHIBIT 1



US00689265B2

(12) **United States Patent**
Heller et al.(10) **Patent No.:** **US 6,689,265 B2**
(45) **Date of Patent:** ***Feb. 10, 2004**(54) **ELECTROCHEMICAL ANALYTE SENSORS
USING THERMOSTABLE SOYBEAN
PEROXIDASE**(75) **Inventors:** Adam Heller, Austin, TX (US); Gregg
L. Kenausis, Austin, TX (US); Qiang
Chen, Austin, TX (US); Mark S.
Vreeke, Alameda, CA (US)(73) **Assignee:** TheraSense, Inc., Alameda, CA (US)(*) **Notice:** Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 131 days.This patent is subject to a terminal dis-
claimer.(21) **Appl. No.:** 09/815,747(22) **Filed:** Mar. 23, 2001(65) **Prim Publication Data**

US 2001/0017269 A1 Aug. 30, 2001

Related U.S. Application Data(63) Continuation of application No. 09/291,230, filed on Apr.
13, 1999, now abandoned, which is a continuation of appli-
cation No. 08/798,596, filed on Feb. 11, 1997, now Pat. No.
5,972,199, which is a continuation-in-part of application No.
08/540,769, filed on Oct. 11, 1995, now Pat. No. 5,665,222.(51) **Int. Cl.** G01N 27/327(52) **U.S. Cl.** 204/403.09; 204/403.04;
204/403.14(58) **Field of Search** 204/403.04, 403.09,
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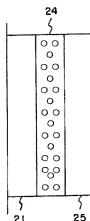
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Primary Examiner—Alex Nogueroles(74) **Attorney, Agent, or Firm**—Merchant & Gould P.C.

(57)

ABSTRACTA sensor for the detection and measurement of an analyte in
a biofluid. The sensor includes two enzymes. One type of
sensor measures the concentration of hydrogen peroxide
using a thermostable peroxidase enzyme that is immobilized
in a redox hydrogel to form a sensing layer on a working
electrode. This sensor also includes a hydrogen peroxide-
generating second enzyme which is insulated from the redox
hydrogel and electrode. This second enzyme generates
hydrogen peroxide in response to the presence of an analyte
or analyte-generated compound. The second enzyme may be
insulated from the electrode by placement of an electrically
insulating layer between the sensing layer and the second
enzyme layer.Alternatively, the second enzyme is immobilized in an
inorganic polymeric matrix, preferably made using a sol-gel
polymerization process. Such matrices include those made
of silica. Often, the second enzyme is stabilized by immo-
bilization in a sol-gel. Further stabilization of polyelectro-
lytic enzymes can be obtained by immobilizing the enzyme
with a polyelectrolytic polymer in the sol-gel matrix.**20 Claims, 20 Drawing Sheets**

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FIG. 1

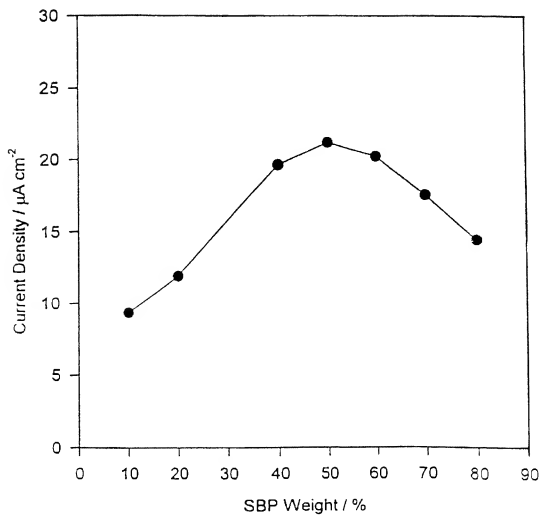


FIG. 2

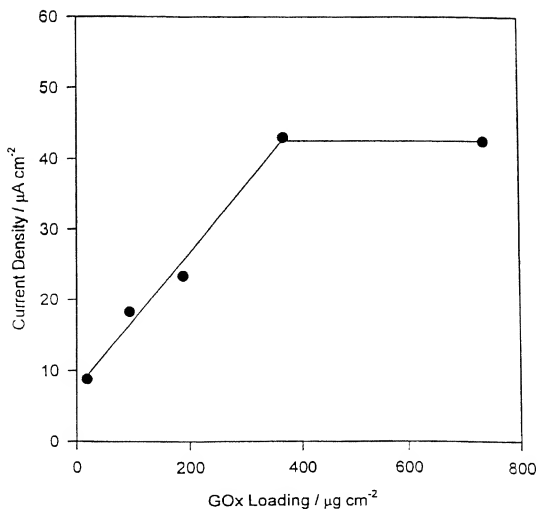


FIG. 3

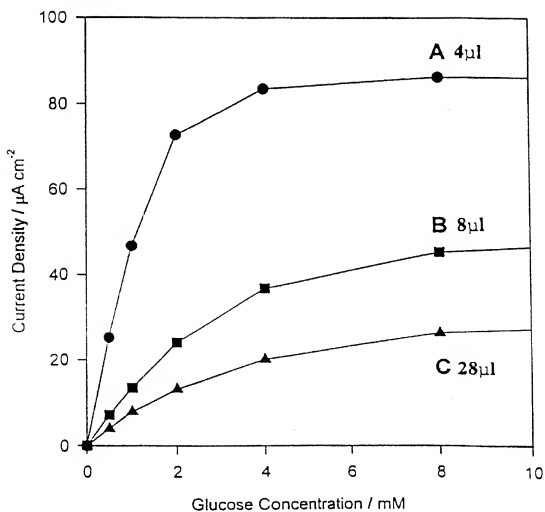


FIG. 4

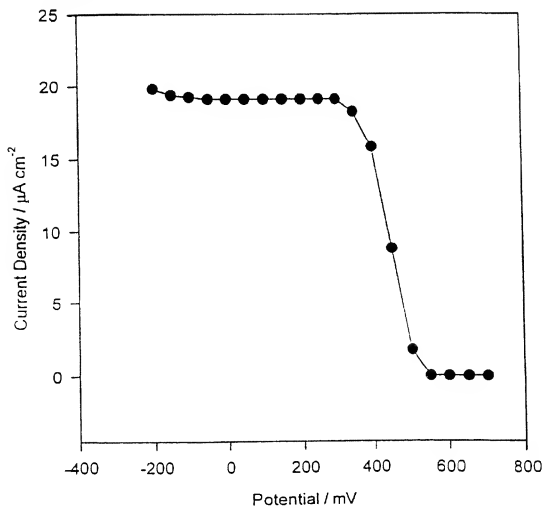


FIG. 5

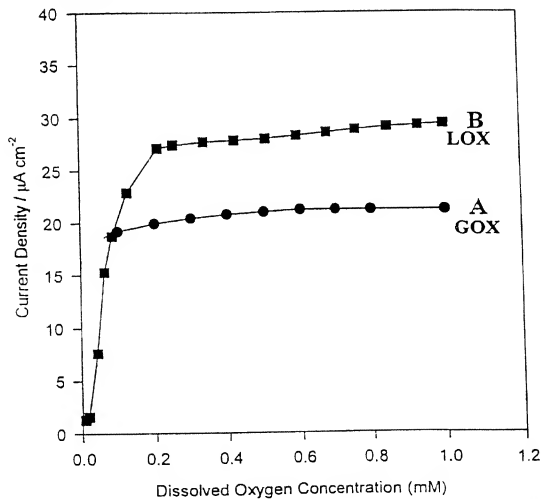
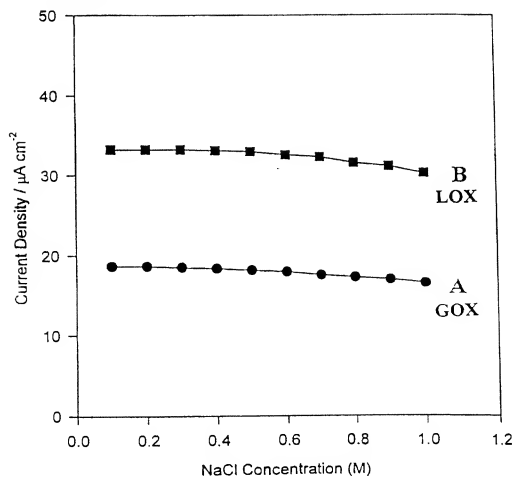


FIG. 6



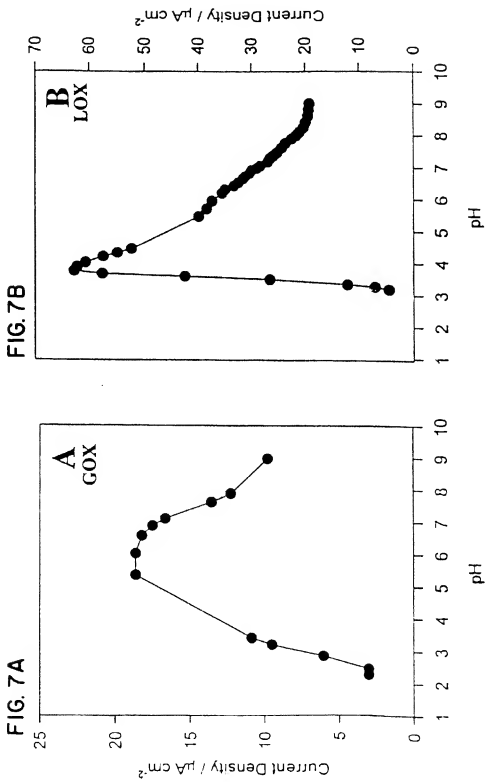
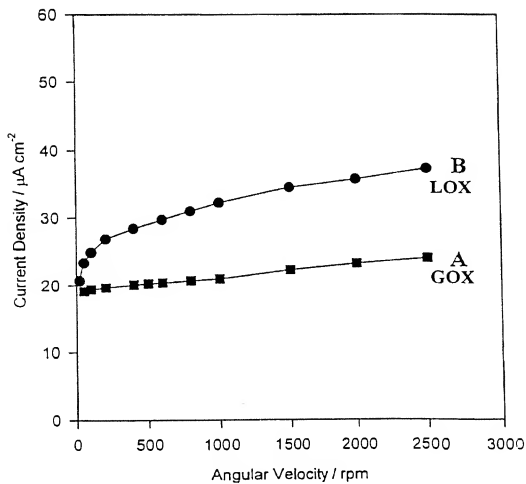


FIG. 8



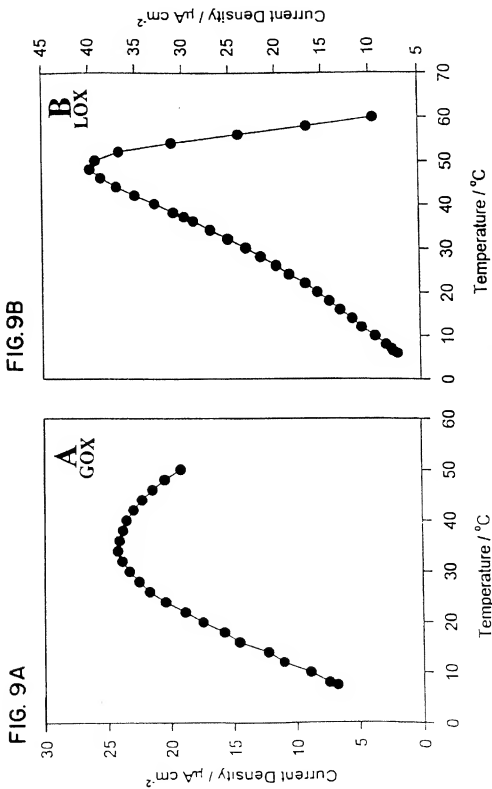


FIG. 10A

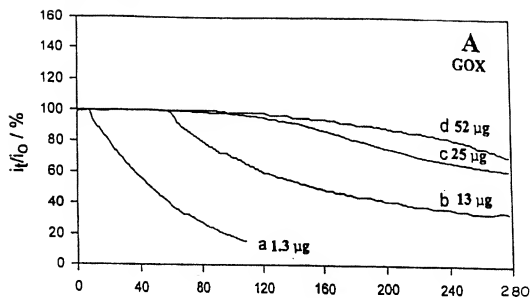


FIG. 10B

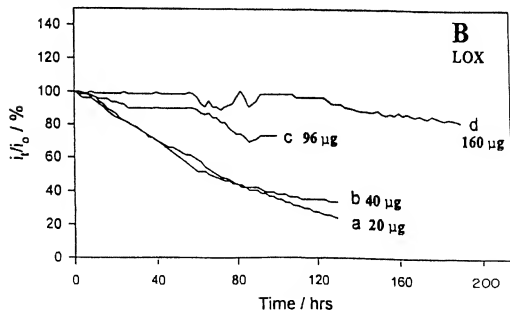


FIG. 11

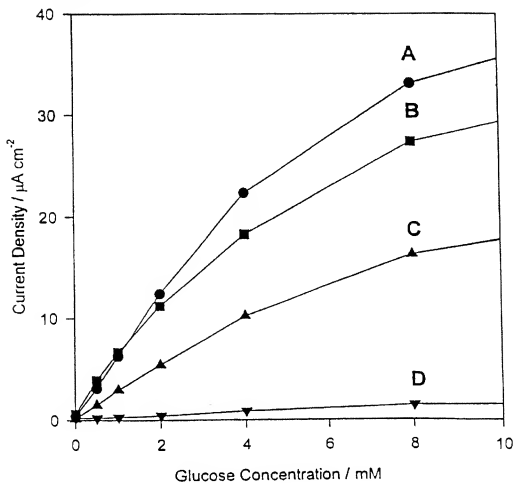


FIG. 12

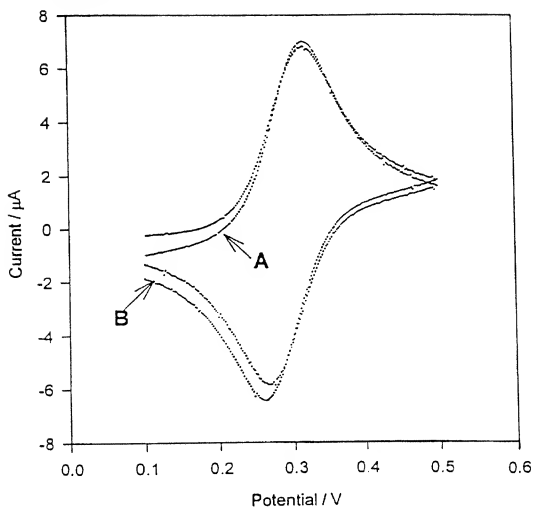
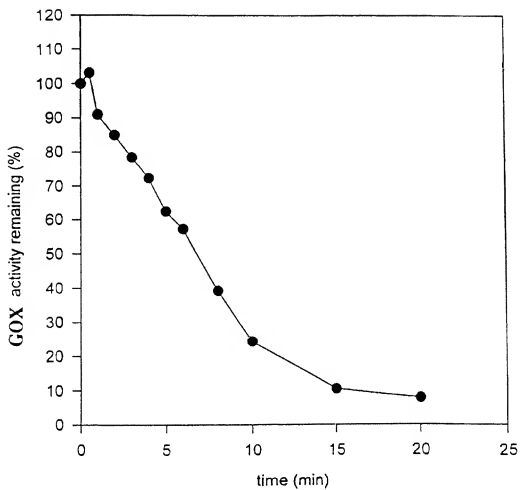


FIG. 13



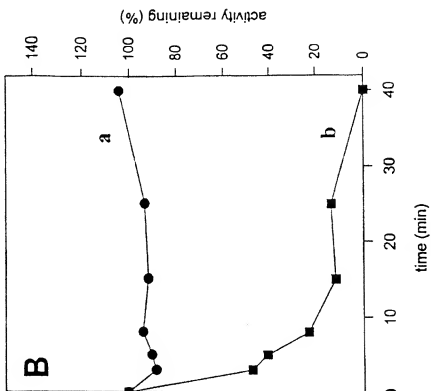


FIG. 14B

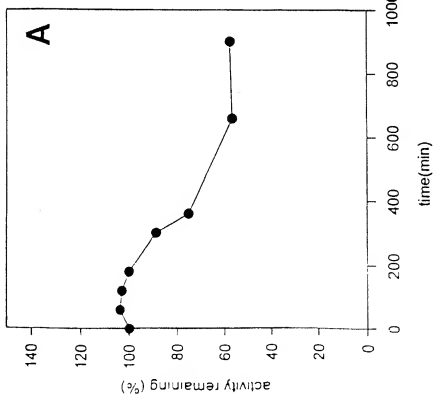


FIG. 14A

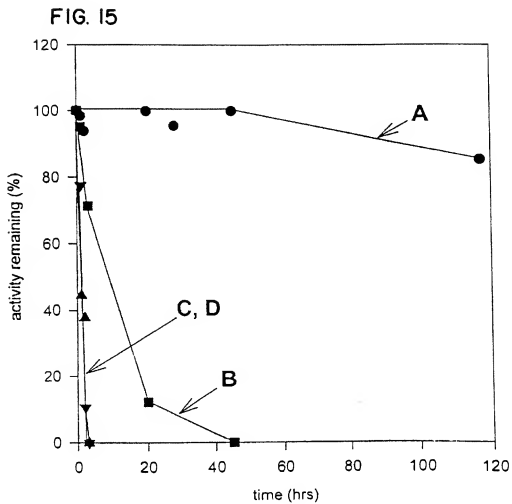


FIG. 16

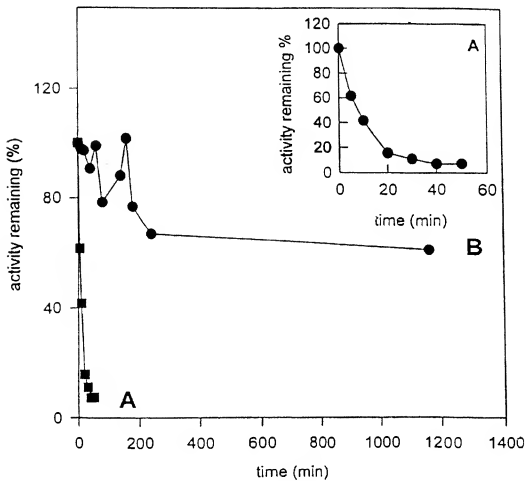


FIG. 17

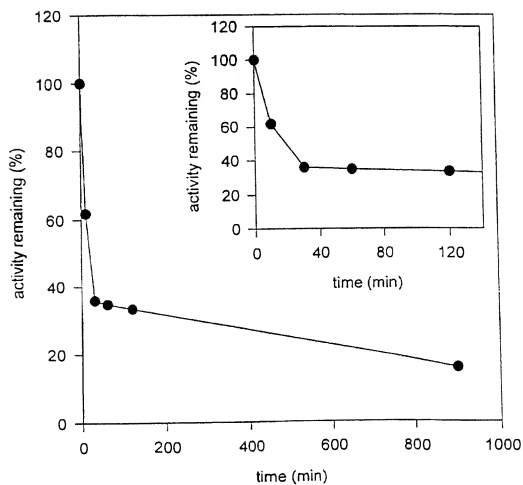


FIG. 18A

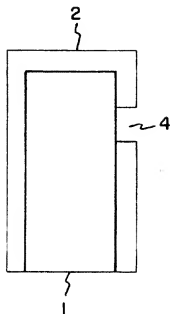


FIG. 18B

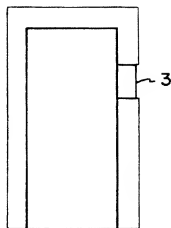


FIG. 19

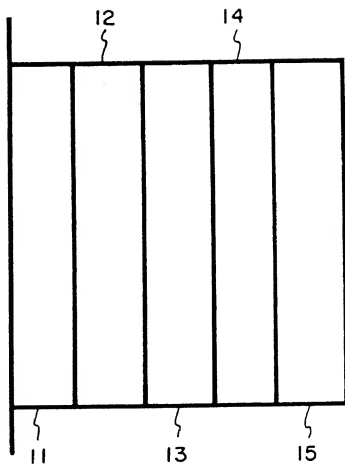


FIG. 21

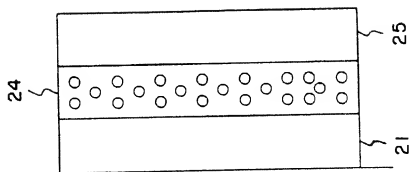
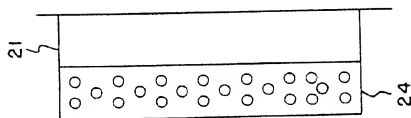


FIG. 20



ELECTROCHEMICAL ANALYTE SENSORS USING THERMOSTABLE SOYBEAN PEROXIDASE

This application is a continuation application under 37 CFR §1.53(b) of Ser. No. 09/291,230, filed on Apr. 13, 1999, now abandoned, which is a continuation of U.S. patent application Ser. No. 08/798,596 filed on Feb. 11, 1997, U.S. Pat. No. 5,972,199, which is a continuation-in-part of U.S. patent application Ser. No. 08/540,789 filed Oct. 11, 1995, U.S. Pat. No. 5,665,222, which application(s) are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to thermostable analyte sensors. More particularly, the invention relates to electrochemical sensors for the measurement of bioanalytes such as glucose and lactate. The inventive sensors include a thermostable peroxidase, such as soybean peroxidase, disposed on an electrode in a redox-compound-containing film. The sensor further includes a hydrogen peroxide-producing enzyme. The inventive sensors, for example in the measurement of blood glucose or lactate, operate for five days or more at 37° C., losing less than 10% of their sensitivity during continuous or intermittent operation.

BACKGROUND OF THE INVENTION

The assay of biochemicals, such as glucose and lactate, is important in medicine, biotechnology, and food processing (dairy and wine). Monitoring of glucose concentrations in fluids of the human body is of particular relevance to diabetes management. Monitoring of lactate in fluids of the human body is of relevance to diagnosis of trauma, of myocardial infarction, congestive heart failure, pulmonary edema, septicemia, hemorrhage, and others. Blood lactate levels above 7–8 mM are indicative of a fatal outcome. Bedside analyzers of lactate are useful in determining the response of patients to treatment, while in accidents and battle, they are useful in triage. Glucose assays are common in clinical practice and are applied in the diagnosis of Diabetes Mellitus and its management. Continuously or intermittently operating glucose sensors, including sensors implanted in the human body, are sought for the management of Type I diabetes, for example, for warning of imminent or actual hypoglycemia and its avoidance. Hypoglycemia can be fatal. For maintenance of diabetic patients at or near normal blood glucose levels, frequent or continuous monitoring of glucose is needed. Today, most Type I diabetic patients maintain their blood glucose at higher than normal levels, so as to reduce risk of fatal hypoglycemia. This is undesirable, as maintenance of higher than normal blood glucose levels has been shown to be a leading cause of blindness, kidney failure, neuropathy, and other complications of diabetes. It would, therefore, be useful to provide a glucose sensor that operates continuously or intermittently for a prolonged period of time to measure glucose in body fluid at 37° C. without substantial loss of sensitivity.

The present invention discloses material, structures, and methods enabling continuous operation of electrochemical sensors, for example, measuring glucose for more than one week or measuring lactate for more than 100 hours, with less than 10% loss in sensitivity.

SUMMARY OF THE INVENTION

Novel electrochemical sensors are presented, some of which are capable of operating at 37° C. continuously or

intermittently, measuring biochemicals in body fluids with less than 10% loss of sensitivity in more than 100 hours of operation as described herein. The inventive sensors are relatively insensitive to electrooxidizable interferents, including ascorbate and acetaminophen.

The sensors of the invention include at least two enzymes, a thermostable peroxidase, such as soybean peroxidase, and a peroxidase-generating enzyme. In a preferred embodiment, redox centers of a thermostable peroxidase are electroreduced by electrons transported from a working electrode through a redox hydrogel in which the thermostable peroxidase is immobilized, preferably at a potential negative of 0.4 V versus the standard calomel electrode (SCE) and positive of –0.15 V (vs SCE). Most preferably, the thermostable peroxidase is coated on the electrode at a potential near 0.0V (vs SCE).

The preferred redox hydrogel comprises at least 20% by weight of water when in contact with a fluid to be assayed, and its redox centers are not leached by the assay fluid at 37° C. Non-leachable redox centers are bound to a polymer that forms the hydrogel upon water uptake. Preferably, the binding to the polymer is through covalent, electrostatic/ionic, or coordination bonds.

The redox centers of the peroxide-generating enzyme are preferably electrically insulated from the electrode, from the redox centers of the thermostable peroxidase, and from the redox centers of the redox hydrogel. The electrically insulated, peroxide-generating enzyme catalyzes reaction of a biochemical analyte, e.g., glucose or lactate, or a product of the analyte, with molecular oxygen. In the oxidation reaction, oxygen is reduced to hydrogen peroxide (H₂O₂).

The hydrogen peroxide-generating enzyme is preferably stabilized in a matrix. The preferred stabilizing matrices are macromolecular and inorganic. The most preferred matrices include silicon atoms, at least 50% of which are covalently linked to neighboring oxygen atoms, which are formed into a three-dimensional, crosslinked network. Such matrices can be made using a sol-gel polymerization process. The stabilizing matrix optionally includes a second polymer, which functions to further stabilize the insulated, peroxide-generating enzyme.

The peroxide-generating enzyme is preferably positioned behind or immobilized in a polymer that is at least tenfold, and preferably at least 100-fold, more permeable to oxygen than is the biochemical analyte to be measured. Examples of such polymers include silicone rubbers, produced by cross-linking a poly(dimethyl siloxane) derivative and cellulose acetate.

BRIEF DESCRIPTION OF THE FIGURES

Referring now to the drawings, wherein like reference numerals and letters indicate corresponding structure throughout the several views and where Examples 2–12 are for sensors described in Example 1:

FIG. 1 is a graph showing the dependence of current density on the weight fraction of the thermostable peroxidase in the sensing layer at 60 $\mu\text{g cm}^{-2}$ loading (16 mM glucose, 1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. 2 is a graph showing the dependence of the electroreduction current density on the GOx loading (16 mM glucose, 1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. 3 is a graph showing the dependence of the calibration curves on the thickness of the cellulose acetate layer between the sensing layer and the immobilized glucose oxidase film. (A) 4 μm , (B) 8 μm , and (C) 28 μm of 0.25%

cellulose acetate (16 mM glucose, 1000 rpm, 37° C. pH7.3, in air, 0.00V (SCE));

FIG. 4 is a graph showing the potential dependence of the current density for a glucose sensor according to Example 4 (5 mM glucose, 1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. 5 is a graph showing the dependence of the current density on the dissolved oxygen concentration, according to Example 5 (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE)), (A) 5 mM glucose, (B) 5 mM lactate;

FIG. 6 is a graph showing the dependence of current density on the concentration of NaCl (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE)), (A) 5 mM glucose, (B) 5 mM lactate;

FIG. 7A and FIG. 7B are graphs showing the dependence of current density on pH,

FIG. 7A shows data for 5 mM glucose;

FIG. 7B shows data for 5 mM lactate (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. 8 is a graph showing the dependence of current density on the angular velocity (37° C., pH7.3, in air, 0.00V (SCE)), (A) 5 mM glucose, (B) 5 mM lactate;

FIG. 9A and FIG. 9B are graphs showing the dependence of current density on temperature.

FIG. 9A shows data for 5 mM glucose;

FIG. 9B shows data for 5 mM lactate (1000 rpm, pH7.3, in air, 0.00V (SCE));

FIG. 10A and FIG. 10B are graphs showing the stability of thermostable peroxidase-based glucose and lactate sensors at different oxidase loadings: Figure (a) 1.3 μ g; (b) 13 μ g; (c) 26 μ g; (d) 52 μ g of immobilized glucose oxidase (FIG. 10A); (a) 20 μ g; (b) 40 μ g; (c) 96 μ g; (d) 160 μ g of immobilized lactate oxidase (FIG. 10B) (1000 rpm, 37° C., pH7.3, in air, 0.00V (SCE));

FIG. 11 is a graph showing glucose calibration curves before and after 280 hours of operation at 37° C. in the stability study of Example 8, (A) 26 μ g GOx before stability experiment; (B) 26 μ g GOx after stability experiment; (C) 1.3 μ g GOx before stability experiment; (D) 1.3 μ g GOx after stability experiment (1000 rpm, 37° C. pH7.3, in air, 0.00V (SCE));

FIG. 12 is a graph showing cyclic voltammograms of the glucose sensor before and after 280 hours of operation at 37° C.; (A) after stability experiment; (B) before stability experiment;

FIG. 13 is a graph of the time dependence of lactate oxidase activity in solution at 63° C.;

FIG. 14A and FIG. 14B are graphs showing time dependence of lactate oxidase-doped silica gel powder activity at 63° C.; FIGS. 14A and 14B(a): PVI-lactate oxidase-doped silica gel, FIG. 14B(b): lactate oxidase-doped silica gel;

FIG. 15 is a graph showing time dependence of the activity of lactate oxidase in different environments at 50° C.; (A) PVI-LOx-doped silica gel; (B) LOx-doped silica gel; (C) PVI-LOx in solution; (D) LOx in solution;

FIG. 16 is a graph showing time dependence of the activity of glucose oxidase in different environments at 63° C.; (A), glucose oxidase in solution; (B) glucose oxidase-doped silica gel powder; the inset shows the short term behavior of (A);

FIG. 17 is a graph showing time dependence of PEI-lactate oxidase-doped silica gel powder activity at 63° C.; inset shows the short term behavior;

FIG. 18A is a diagrammatic representation of an electrode;

FIG. 18B is a diagrammatic representation of an electrode with a sensing layer.

FIG. 19 is a diagrammatic representation of a sensing layer of a biosensor of the invention;

FIG. 20 is a diagrammatic representation of a two-layered sensing layer of a thermostable biosensor of the invention; and

FIG. 21 is a diagrammatic representation of a three-layered sensing layer of a thermostable biosensors of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Referring to the drawings in general and FIGS. 18(a)-(b) in particular, the sensors of the invention typically include a non-corroding working electrode 1 which is substantially covered by an insulator 2 having a recess 4 to allow electrolytic contact between an analyte-containing fluid and the working electrode 1. A film 3, having a plurality of layers, is positioned within recess 4. FIGS. 19-21 show three embodiments of film 3. In general, film 3 includes a sensing layer 11, 21 and a second enzyme layer 13, 24. Film 3 may also contain one or more optional layers, such as an electrically insulating layer 12 between the sensing layer 11 and second enzyme layer 13, an analyte-transport controlling layer 14 to control the flow of analyte to the sensing layer 11, and a biocompatible layer 15, 25 for biocompatibility of the sensor in body fluids.

Electrodes

The electrochemical sensors of the invention include at least two electrodes for contacting the electrolytic solution to be assayed. One of these electrodes is a working electrode 1, which is made from non-corroding metal or carbon. A carbon working electrode may be vitreous or graphitic and can be made from a solid or a paste. A metallic working electrode may be made from platinum group metals, including palladium or gold, or a non-corroding metallically conducting oxide, such as ruthenium dioxide. The working electrode may be a wire or a thin conducting film applied to a substrate, for example, by coating or printing.

Typically, only a portion of the surface of the metallic or carbon conductor is in electrolytic contact with the analyte-containing solution. This portion is called the working surface of the electrode. The remaining surface of the electrode is isolated from the solution by an insulator 2. Examples of useful insulators include polymers such as polyimides, polytetrafluoroethylene, polyhexafluoropropylene and silicones, also known as polysiloxanes.

In addition to the working electrode, the sensors of the invention also include a reference electrode or a combined reference and counter electrode (also termed a quasi-reference electrode or a counter/reference electrode). The reference or counter/reference electrode may be, for example, a silver/silver chloride electrode. If the sensor does not have a counter/reference electrode then it will include a separate counter electrode, which may be made from the same materials as the working electrode.

Sensors of the present invention have one or more working electrodes and one or more counter, reference, and/or counter/reference electrodes. One embodiment of the sensor of the present invention has two or more working electrodes. These working electrodes may be integrally connected or they may be kept separate.

Typically, for *in vivo* use the working electrodes of the present invention are implanted subcutaneously in the skin of a patient for direct contact with the body fluids of the

patient, such as blood. When multiple working electrodes are used, they may be implanted together or at different positions in the body. The counter, reference, and/or counter/reference electrodes may also be implanted either proximate to the working electrodes or at other positions within the body of the patient. Alternatively, the counter, reference, and/or counter/reference electrodes may be placed on the skin of the patient.

Sensing Layer

The portion of the working electrode that is not insulated is coated with a sensing layer 3. The typical sensing layer 3 of the sensors of the present invention includes a redox polymer which is capable of swelling in water to form a redox hydrogel. Sensing layer 3 also typically includes an enzyme which is capable of catalyzing a reaction of the analyte or an analyte-generated compound. Preferably, that enzyme is immobilized in the redox polymer. In one embodiment of the invention, the sensor contains a peroxidase which is preferably a thermostable peroxidase such as soybean peroxidase. The peroxidase-containing hydrogel forms a sensing layer that transduces a flux of H_2O_2 into an electrical signal.

The redox polymer contains redox centers that are bound to the polymer by covalent, coordinative, or electrostatic bonds; the latter resulting preferably from interactions of charged sites of the polymer with an oppositely charged redox species. Preferably, the redox mediator is covalently or coordinatively bound to the redox polymer, although it may also be immobilized by entrapment or by electrostatic bonding.

The redox centers are preferably non-leachable. For redox species electrostatically bound to the polymer, the absence of leaching generally requires that the charged redox species has, at the pH of the analyte solution, a charge of at least two, and preferably three or more. For example, if the redox species is a cation, it preferably has at least a +2 charge, and is preferably +3 or better. The greater the charge of the redox species, the slower the leaching. Similarly, if the redox species is an anion and the binding to the polymer is electrostatic, then it should have a -2 charge and is preferably -3 or better. Preferably, most of the redox centers (at least about 90%) remain electrode-bound for at least 14 days at 37° C.

The redox polymer is designed to swell in water and dilute aqueous solutions. The water uptake should add at least 10 weight percent, and preferably 20 weight percent, to the weight of the dry polymer. The thermostable peroxidase in the redox polymer layer is not substantially leached by water or by a physiological buffer solution at 37° C. The redox polymer layer containing the thermostable peroxidase can be conveniently made, for example, as described by Gregg and Heller, *J. Phys. Chem.* 95:5970 (1991), in U.S. Pat. No. 5,262,035, and in copending U.S. patent application Ser. No. 08/540,789, filed Oct. 11, 1995.

In general, the redox polymer contains redox centers that are stable and have a redox potential between about -0.1 V (vs SCE) and about +0.6 V (vs SCE). The preferred range is between about -0.1 V (vs SCE) and about +0.4 V (vs SCE). Poising the electrode at a potential where O_2 is not electroreduced is desirable; otherwise the measured signal resulting from the electroreduction of H_2O_2 to water must be corrected for the background caused by electroreduction of dissolved O_2 . Furthermore, it is also desirable to poise the electrode at a potential where the rate of electrooxidation of other interferents, such as urate, ascorbate, and acetaminophen, is not significant, so that correction for their electrooxidation will not be required.

Examples of useful redox centers that can be bound to the polymer of the sensing layer are described in U.S. Pat. Nos. 5,264,104; 5,356,786; 5,262,035; and 5,320,725, herein incorporated by reference. Typically, the redox mediators are metal complexes, particularly of osmium, ruthenium, iron and cobalt, or organic redox compounds, such as quinones and other compounds having a quinoid structure. The preferred redox centers exchange electrons rapidly with each other. One example of the preferred redox centers are osmium transition metal complexes with one or more ligands having a nitrogen-containing heterocycle such as 2,2'-bipyridine, 1,10-phenanthroline or derivatives thereof. In particular, it has been determined that osmium cations complexed with two ligands containing 2,2'-bipyridine, 1,10-phenanthroline, or derivatives thereof, the two ligands not necessarily being the same, form particularly useful redox centers in the sensors of the present invention. Preferred derivatives of 2,2'-bipyridine for complex formation with the osmium cation are 4,4'-dimethyl-2,2'-bipyridine and mono-, di-, and polyalkoxy-2,2'-bipyridines such as 4,4'-dimethoxy-2,2'-bipyridine, where the carbon to oxygen ratio of the alkoxy groups is sufficient to retain solubility of the transition metal complex in water.

One useful group of redox polymers is derived from poly(4-vinyl pyridine) and is made by complexing between one third and one fifteenth of the pyridine rings with $[Os(bpy)_2Cl]^{2+}$ where bpy is 2,2'-bipyridine, or with $[Os(phen)_2Cl]^{2+}$ where phen is 1,10-phenanthroline, and where, preferably, some of the pyridine rings of the polymer are quaternized by reaction with 2-bromocetylamine. Useful polymers are also derived from poly(N-vinyl imidazole) (termed PVI), poly(acrylamide co-4-vinylpyridine), and poly(acrylamide co-4-vinylimidazole).

To produce the inventive sensors, a hydrogen peroxide sensing layer, containing a thermostable peroxidase, is prepared by the dropwise mixing of solutions of the peroxidase, the redox polymer, and a crosslinker on a clean electrode surface. These solutions are allowed to dry and cure. The solutions may also be premixed, and a droplet of the premixed solution may be placed on the electrode. An example of a useful crosslinker is poly(ethylene glycol) diglycidyl ether with a molecular weight between 300 and 600, preferably 400 and 600. Another useful crosslinker is the bis N-hydroxysuccinimide ester of poly(ethylene glycol)- α,ω -dicarboxylic acid.

Second Enzyme Layer

A second enzyme layer 13 on the working electrode catalyzes the reaction of O_2 with a substrate whereby H_2O_2 is produced. The substrate is generally the analyte to be assayed by the sensor. Alternatively, the substrate is a reaction product of the analyte to be assayed, for example the product of hydrolysis of the analyte. The second enzyme used in layer 13 is also termed herein the peroxide-generating enzyme. In the inventive sensors, the redox centers of the peroxide-generating enzyme are prevented from being reduced or oxidized directly by electron exchange with the redox polymer of the sensing layer. Prevention of such oxidation or reduction is accomplished by methods discussed hereinbelow.

First, prevention of oxidation/reduction of the peroxide-generating (second) enzyme by the redox polymer can be intrinsic to the second enzyme. For example, the second enzyme may include a sufficiently thick, natural, electrically insulating protein or glycoprotein layer over its reaction center or centers. Such a layer prevents electron transfer to or from the redox polymer. In this case, no further means for preventing reduction or oxidation of the second enzyme by the redox polymer is required.

Inorganic Polymeric Matrix Immobilization and Stabilization

Alternatively, the H_2O_2 -generating (second) enzyme is immobilized in a non-conducting inorganic or organic polymeric matrix to prevent electron exchange between its redox centers and the redox polymer. The immobilizing matrix is preferably highly permeable to O_2 and usually is more permeable to O_2 than to the analyte or to its precursor. The preferred immobilizing matrices are those in which the second enzyme is stabilized, becoming itself thermostable through immobilization, as discussed more fully below.

The sol-gel polymerization process provides a method for the preparation of an inorganic polymeric matrix (e.g., glass) by the polymerization of suitable monomers at or near room-temperature. Suitable monomers include alkoxides and esters of metal and semiconducting elements, with preferred metals and semiconductors comprising Si, Al, Ti, and Pb. The most preferred monomers include silicon and have a silicon to oxygen ratio of between about 1:2 and about 1:4.

Enzymes can be immobilized in silica and other inorganic polymeric matrices made by sol-gel processes, involving, for example, the hydrolysis of tetramethoxysilane or other polyalkoxysilane that may contain one or more silicon atoms. Condensation of the resulting silanol in the presence of the enzyme results in entrapment of the enzyme. This process has been referred to as sol-gel immobilization.

Enzymes can be immobilized in inorganic polymeric matrices to prevent electrical contact between the immobilized second enzyme and the redox polymer and/or electrode. Furthermore, binding of enzymes in silica or other inorganic polymeric matrices formed from sol-gels can stabilize the enzyme. Entrapment of glucose oxidase, a glycoprotein, in silica sol-gel greatly improves the stability of the enzyme, which retains activity when heated in water to 98° C. for 10 minutes.

When lactate oxidase, which is not a glycoprotein, was similarly immobilized by the sol-gel process in silica, the enzyme was not particularly thermostable. However, when lactate oxidase is dissolved in an aqueous buffer solution in which poly(N-vinyl imidazole) is co-dissolved, and the lactate oxidase-poly(N-vinyl imidazole) mixture is immobilized in the silica by the sol-gel process, a uniquely stable, immobilized lactate oxidase is obtained. The stabilized lactate oxidase can be heated in water to 90° C. for 10 minutes and still retain enzymatic activity.

Poly(N-vinyl imidazole), a polycation at pH 7, binds at this pH to enzymes such as lactate oxidase, that are polyanions at pH 7. Thus, the addition of a particular polymer to a particular enzyme can greatly increase the stability of the enzyme. In the case of lactate oxidase, addition of polyethyleneimine, also a polycationic polymer and also multiply protonated at pH 7, in place of poly(N-vinyl imidazole) improved stability of the enzyme, although not as much as the addition of the preferred polymer, poly(N-vinyl imidazole).

If the enzyme is a polycation or polyanion at a particular pH, a polymeric polyanion or polycation, respectively, can be coimmobilized in an inorganic polymeric matrix formed from a sol-gel to stabilize the enzyme. The stabilized enzyme can then be used at higher temperatures and/or for longer durations than would be possible if the enzyme were immobilized alone in the sol-gel.

After gelling, the silicas, in which the peroxide-generating, now thermostable, second enzyme is immobilized, are hard materials that can be ground into fine powders. These powders are then dispersed in highly

O_2 -permeable polymers, further discussed hereinbelow, to form a peroxide-generating second layer 13 of the inventive sensors. The peroxide-generating layer 13 preferably has the following desired properties: thermostability; absence of electron transfer between the redox polymer of the sensing layer and the redox centers of the enzyme of the second enzyme containing H_2O_2 -generating layer, i.e. absence of oxidation or reduction of the redox polymer by redox centers of the second enzyme; and high O_2 permeability.

Alternatively, when electron transport between the redox polymer of the sensing layer and the peroxide-generating second enzyme is avoided because of intrinsic electrical insulation of the H_2O_2 generating enzyme or by immobilization of the enzyme in an electrically insulating matrix (such as the inorganic polymeric matrices described above), it is practical to incorporate the second enzyme in the peroxidase-containing sensing layer. In this case, the sensing layer includes the redox polymer, electrically connecting the thermostable peroxidase (first enzyme) to the electrode, and also includes the peroxide-generating enzyme (second enzyme), the redox centers of which are not electrically connected to the redox polymer or to the electrode.

Alternatively, the peroxide-generating enzyme layer 24 can be formed directly over the sensing layer 21 (see FIGS. 20 and 21), but only when the peroxide-generating enzyme is intrinsically insulated or immobilized in an electrically insulating matrix. If the H_2O_2 generating enzyme is neither intrinsically insulating nor immobilized in an insulating matrix, an H_2O_2 -permeable, electrically insulating layer 12 can be placed between the sensing layer 11 and the peroxide-generating (second) enzyme layer 13 to achieve the desired insulation.

The peroxide-generating (second) enzyme layer is formed by any of the following methods. Particles, such as silica-containing particles in which the second enzyme is stabilized, can be dispersed in a polymer, such as a silicone rubber solution or silicone rubber precursor solution. A droplet of this mixture is then applied to the working electrode. Alternatively, the peroxide-generating layer is formed by adding a cross-linking agent to the second enzyme and applying a droplet containing the second enzyme and its crosslinker to the working electrode. Useful cross-linking agents include, for example, poly(ethylene glycol) diglycidyl ether (PEGDGE). For cross-linking of glycoproteins such as glucose oxidase, the enzyme is oxidized with $NaIO_4$, forming reactive aldehyde functions condensing with surface amines, such as those of enzyme lysines. The preferred method for producing a peroxide-generating layer containing glucose oxidase, when the enzyme is not immobilized in a silica sol-gel, is the latter, $NaIO_4$ method. For peroxide-generating layers containing lactate oxidase, which is also not immobilized in a silica sol-gel, cross-linking of its mixture with poly(N-vinyl imidazole) with PEGDGE is preferred. The second enzyme may also be immobilized with glutaraldehyde or glutaraldehyde and a protein such as albumin.

An enzyme stabilized by the silica sol gel matrix can be ground to a fine powder and dispersed in a silicone, preferably in an elastomeric silicone, and most preferably in a water-based elastomeric silicone precursor. This dispersion is then applied to the thermostable peroxidase layer to form a hydrogen peroxide-generating layer. Silicone is a preferred binder in this layer due to its oxygen permeability. Electrically Insulating Layer

Another means for preventing oxidation/reduction of the peroxide-generating (second) enzyme is the addition of an electrically insulating membrane 12 that is permeable to

H₂O₂ between sensing layer 11 and second enzyme layer 13. The insulating membrane can be inorganic or organic, but preferably is an organic polymer such as cellulose acetate. The second enzyme may be immobilized in a layer 13 over the polymeric membrane.

Preferably, the insulating membrane 12 is permeable to the small H₂O₂ molecule but is not permeable to the larger, readily oxidizable, potentially interfering ascorbate, acetaminophen or urate species. Preferably, the ratio of the permeability of layer 12 to H₂O₂ and one or more of ascorbate, acetaminophen, and urate is greater than 2:1. Useful, selectively H₂O₂ permeating polymer films include dense cellulose acetate, cured Nafion™, and nylon. Using known, standard methods, dense, small pore size films of cellulose acetate or nylon are cast from cyclohexanone or tetrahydrofuran; Nafion™ films are made from alcohol and alcohol-water emulsions and curing.

Analyte-Transport Controlling Layer

If the second enzyme is not immobilized, or when it is desired to broaden the dynamic range of the sensor so that the sensor will respond, preferably linearly, to higher concentrations of the analyte, then an analyte-transport controlling layer 14 is placed between the solution that is being analyzed and second enzyme layer 13 of the sensor. This analyte-transport controlling layer 13 functions to contain the second enzyme and thereby avoid its loss, and/or to limit the flux of the analyte or second enzyme substrate to the second enzyme layer.

It is of essence that this second membrane be permeable to O₂, the co-reactant in the enzyme-catalyzed generation of H₂O₂, in the presence of the substrate. It is also of essence that the ratio of permeabilities of O₂ and of the substrate or its precursor through this second membrane be large enough for oxidation or dehydrogenation of the substrate in the reaction catalyzed by the second enzyme with the concurrent formation of water, a reaction whereby O₂ is consumed. Because the concentration of O₂ in saturated aqueous solutions is well below the usual concentrations of analytes such as glucose or lactate in biological fluids, including fluids in the body of animals, analyte-transport controlling layers 14 that are much more permeable to O₂ than to the substrate or its precursor are preferred.

Generally, membranes or polymers for which the permeability coefficient of molecular oxygen is greater than 10⁻⁸ cm³ (STP) cm cm⁻² s⁻¹ (cm Hg)⁻¹ at 25° C. are preferred. (Explanation of the permeability units is given in *Polymer Handbook, Second Edition*, J. Pandrup and E. H. Immergut, Editors, John Wiley & Sons, New York, 1975, pages III-229-231.) Polymers for use in the analyte-transport controlling layer 14 are those that are highly permeable to O₂, preferably those in which the ratio of O₂ and substrate (or substrate precursor) permeabilities is greater than 10:1, and more preferably greater than 100:1. It is well known that the permeability of O₂ in silicones, for example in silicone rubbers, formed by cross-linking poly(dimethyl siloxanes), is particularly high. Thus, silicones are useful polymers for this layer. Another useful material is cellulose acetate.

Biocompatible Layer

For in vivo use, the sensor is preferably coated with a biocompatible film 15 that is permeable to the substrate converted in the reaction catalyzed by the second enzyme. This biocompatible film is placed on the solution side of the sensor.

The preferred biocompatible layer 15 is formed of a hydrogel, e.g., a polymeric composition which contains more than 20% by weight of water when in equilibrium with a physiological environment such as living tissue or blood.

An example is a cross-linked derivative of polyethylene oxide, e.g., a tetraacrylated derivative of polyethylene oxide. The polymeric compositions are non-toxic, non-immunogenic, non-thrombogenic, and otherwise compatible with living tissue of animals.

A biocompatible layer 15 is not necessary if the analyte-transport controlling layer 14 or the second enzyme layer 13 are biocompatible. For example, many solid silicones are biocompatible and therefore require no additional biocompatible layer (see FIG. 20, in contrast the embodiment of the invention shown in FIG. 21 includes a biocompatible layer 25).

Stability of the Sensor

An important performance criterion of the inventive sensor is the rate of loss of sensitivity. This rate depends on the concentration of the analyte, because loss in sensitivity is usually first noticed at high analyte concentrations. At higher analyte concentrations, where complete conversion of the incoming analyte flux to H₂O₂ requires the presence of more of the active second enzyme, the system is very sensitive to inadequate substrate conversion in the second enzyme layer. The rate of loss of sensitivity can be reduced, even when the second enzyme is not particularly thermostable, by limiting the flux of analyte to the second enzyme layer 13 by using an analyte-transport controlling layer 14 and limiting thereby the analyte concentration.

Loss of sensitivity can also be limited and even avoided for a period of days or weeks, by incorporating in the second enzyme layer a large amount of enzyme that even if part of it becomes inactive, all of the analyte flux is still transduced by the remaining second enzyme into an H₂O₂ flux. If a thermostable second enzyme, such as an enzyme entrapped in an inorganic polymeric matrix, is used to form the second enzyme layer 13, sensors of unprecedentedly good stability and long life can be built and can be further improved either by incorporating a large excess of the thermostable second enzyme, much more than needed to convert the analyte flux into an H₂O₂ flux, or by limiting the analyte flux, or by using both techniques cooperatively.

The preferred sensors are also stable for long periods of time at temperatures of 37° C. or greater. Preferably, the sensor is stable for 100 hours or more at this temperature with a drop in the sensor's output of 10% or less. Preferably, the sensor and the enzymes within the sensor are stable at 30° C., more preferably at 40° C., and most preferably at 50° C. or 60° C. or higher.

Other Sensors

Many of the principles of the invention can be used in sensors other than the peroxidase sensors described hereinabove. In particular, the immobilization of a second enzyme in an inorganic polymeric matrix formed using the sol-gel process can be used effectively in the enzyme sensor described in U.S. Pat. No. 5,593,852 herein incorporated by reference. In the sensor, a layered structure similar to that depicted in FIGS. 19-21 is used. However, in the sensing layer an analyte-sensitive enzyme is used rather than a peroxidase. For example, glucose oxidase or glucose dehydrogenase is present in the sensing layer if the analyte is glucose or lactate oxidase is used if the analyte is lactate.

These sensors often have a second enzyme layer which functions as an interferent-eliminating layer. This layer contains one or more enzymes which catalyze reactions of interferents, such as ascorbate, urate, acetaminophen. Enzymes for use in this layer include peroxidases and lactate oxidase (when glucose is the analyte) or glucose oxidase (when lactate is the analyte). These enzymes can be immobilized in an inorganic polymeric matrix as described here-

inabove and used to form a second enzyme layer. Furthermore, these enzymes may be stabilized by immobilization in the inorganic polymeric matrix either alone or in conjunction with a polyelectrolytic polymer as described hereinabove.

EXAMPLES

The invention will be further defined by reference to the following Examples. These examples are not intended to limit the scope of the invention, but rather to illustrate some of the invention's specific embodiments.

Example 1

Preparation of 4-layered Sensors

Soybean peroxidase (SBP) was obtained from Harlan Bioproducts for Science, Indianapolis, Ind., High grade, 130 pyrogallol units/mg (Cat. No: SP04 Lot No: SPL0515). The lactate oxidase (LOx) was obtained from Genzyme, Boston, Mass., Lot # D50293, Cat. # 70-1381-01, EC 1.1.2.3 from *Aerococcus viridans*, 37.0 units/mg of powder. Poly(ethylene glycol) diglycidyl ether (PEGDGE) was obtained from Polysciences, Inc., Warrington, Pa. A 30% hydrogen peroxide solution was obtained from Aldrich, and diluted solutions were prepared daily.

Electrodes

Vitreous carbon electrodes, 3 mm in diameter, were polished and cleaned using 3 grades of alumina slurry: 5, 1, and 0.3 microns, with sonication and rinsing between grades. Each polished and cleaned electrode was tested in PBS by scanning the potentials of interest (-0.4 V to +0.4 V vs. SCE) to ensure that the electrochemistry was featureless. The four-layered sensors were prepared as follows:

Hydrogen Peroxide Sensing Layer

A redox polymer, PVP-bpy-Os (or POs-EA), was synthesized by partially complexing the pyridine nitrogens of poly(4-vinylpyridine) with Os(bpy)₂Cl^{+/2}, and then partially quaternizing the resulting polymer with 2-bromoethylamine, by the method described in Gregg and Heller, 1991, *J Phys. Chem.* 95:5970. The osmium-containing redox centers allow for electrical communication between the carbon surface and the peroxidase heme centers, and the pyridinium-N-ethylamine functions enhance hydration and provide primary amines for cross-linking. The ratio of unmodified pyridines to osmium-complexed pyridines to ethylamine quaternized pyridines was 3.3:1.0:0.8.

The first layer, the hydrogen peroxide sensing layer, was prepared by placing droplets of 2 μ l PVP-bpy-Os (5 mg/ml), 2 μ l soybean peroxidase (5 mg/ml), and 1 μ l of the crosslinker PEGDGE (2.5 mg/ml) on the carbon surface. The droplets were mixed on the surface of the electrode with the tip of a syringe. The electrode was then allowed to dry for 16 hours at room temperature. The resulting dark purple film appeared to be well spread and uniform. The dried electrode was washed in PBS for 20 minutes at 1000 rpm, rinsed with water, and permitted to dry at room temperature.

Hydrogen Peroxide Transport-Limiting Layer

To prepare the second layer, 4 μ l of 0.5% cellulose acetate (Sigma, approximately 40% acetate content) in cyclohexanone (Aldrich) was placed atop the dried and washed hydrogen peroxide sensing layer. The electrode was permitted to dry for two hours at room temperature. The resulting film appeared to be well spread and uniform.

Second Enzyme Layer (Without Sol-gel Silica)

To immobilize the glucose oxidase (GOx) enzyme, two different immobilization procedures were used. For GOx, a solution of 100 μ l of 20 mg/ml of GOx in water was

prepared. Glucose oxidase (GOx) was obtained from Sigma, ED 1.1.3.4 from *Aspergillus niger* type X-S, 198 units/mg solid, 75% protein. After the addition of 50 μ l of 12 mg/ml NaIO₄ in water, the enzyme-periodate solution was incubated at room temperature and in the dark for two hours, following the procedure described in Vreeke et al., 1995, *Anal Chem.* 67:4247. A volume of 4 μ l of the incubated mixture was placed atop the cellulose acetate film to form an immobilized GOx analyte sensing layer, which was permitted to dry overnight.

For LOx, a mixture of 10 μ l of 20 mg/ml LOx, 20 μ l of 10 mg/ml PVI, and 10 μ l of 5 mg/ml PEGDGE in water was prepared. A volume of 4 μ l of the LOx mixture was placed atop the cellulose acetate film to form an immobilized LOx analyte sensing layer, which was permitted to dry overnight.

Analyte Transport-Limiting Layer

The fourth polymeric layer, an analyte transport-limiting layer of cellulose acetate, was prepared and applied to the analyte sensing layer as described above for the hydrogen peroxide transport-limiting layer. A volume of 4 μ l of 0.5% cellulose acetate in cyclohexanone was placed atop the immobilized oxidase layer (analyte sensing layer), and permitted to dry for two hours.

The four-layered electrode was then washed in PBS for 25 minutes and rinsed with water.

Example 2

Varying Amounts of Soybean Peroxidase

The 4-layered electrodes were prepared as described for Example 1, but varying the enzyme weight fraction of soybean peroxidase in the crosslinked redox polymer of the hydrogen peroxide sensing layer or by varying the total loading of SBP. Current measurements were collected using a Princeton Applied Research model 273 potentiostat/galvanostat in a 3-electrode cell. All measurements were performed using a 20 mM phosphate buffer (pH 7.3) containing 0.1 M NaCl, except measurements in which pH dependence was studied. In experiments where pH was varied, 2M solutions of HCl or NaOH were added to the phosphate buffer solution.

Glucose and lactate solutions were prepared by diluting a stock 2M solution in phosphate buffer. All experiments were run at 37° C. under air in 100 ml of phosphate buffer, unless otherwise indicated. The cell contained a rotating glassy carbon working electrode, a saturated calomel reference electrode (SCE), and a platinum counter electrode, isolated from the bulk solution by a Vycor™ frit. The stability measurements were run in a stirred cell, the rotating disk experiments were performed using a Pine RDE4 potentiostat, with an MSRX speed controller, an X-Y-Y' plotter, and a VWR 1165 refrigerated constant temperature circulator. The rotating electrode experiments were performed at 1000 rpm, unless otherwise noted.

Results:

As shown in FIG. 1, for a given mass of redox polymer (POsEA), the current density peaked between 40 wt % and 60 wt % of SBP. For a 50 wt % electrode, the current density reached a plateau at a loading of 60 μ g/cm². Although current densities were independent of loading above 60 μ g/cm², the response times increased with increased loading. For this reason, the preferred sensing layer contained 50 wt % SBP at a total loading of 60 μ g/cm².

FIG. 2 shows the dependence of current densities on the amount of glucose oxidase immobilized on the electrode. The current densities at 4 mM glucose reached a plateau at GOx loadings of 375 μ g/cm². Electrodes made with LOx

exhibited similar behavior with a current density plateau at a loading of 600 $\mu\text{g}/\text{cm}^2$.

Example 3

Varying Thickness of First Transport-controlling Layer and Fourth Analyte-transport-limiting Layer

The hydrogen peroxide transport-controlling layer functions to control the transport of hydrogen peroxide from the analyte sensing layer to the hydrogen peroxide sensing layer. This layer also serves to electrically isolate the POsEA of the hydrogen peroxide sensing layer from the immobilized oxidase layer, that is, to prevent "short-circuiting" of the sensor through "wiring" of the oxidase.

When the immobilized oxidase of the analyte sensing layer is not sufficiently insulated from the hydrogen peroxide sensing layer, redox centers of the oxidase and osmium centers of the POs-EA are electrically connected. This results in the "short-circuiting" of the electrodes. As described in Vreeke and Rocca, 1996, *Electroanalysis* 8:55, a symptom of "short-circuiting", which is often seen only at high substrate concentrations, is the suppression of the catalytic reduction current upon increasing substrate concentration. In the present experiments, "short circuiting" was diagnosed by poisoning the electrode at +0.5 V (SCE), where the hydrogen peroxide was not catalytically electroreduced on the "wired" peroxidase layer, and injecting 10 mM substrate. Flow of an oxidation current indicated that the electrode had a short circuit.

In the electrodes of Example 1 formed with application of 4 μl of 0.25% cellulose acetate, "short circuiting" by "wiring" of the oxidase by POsEA was effectively prevented, in contrast to electrodes prepared with thinner cellulose acetate films. (data not shown). Electrodes made without the inner cellulose acetate layer invariably exhibited short-circuiting, and the effect was often quite substantial. For example, a typical glucose electrode made without this insulating layer showed current suppression at concentrations higher than 1 mM glucose, and the current was suppressed to less than 50% of its maximum value at a concentration of 20 mM. This observation demonstrates the necessity of the insulating layer in this type of sensor.

Membranes, having a range of thickness, were obtained by using solutions of different cellulose acetate concentrations, and applying various volumes of these. The thinnest membranes were made by applying 1 μl of 0.25 weight % cellulose acetate. The thickest membranes were made by applying 4 μl of 0.5 weight % cellulose acetate solution to the surface.

While the presence of the inner cellulose acetate layer effectively prevented short-circuiting, the sensitivity of the electrodes dropped by about 50%. The sensitivity loss is attributed to less efficient collection of the H_2O_2 generated by the oxidase-catalyzed reaction.

An analyte transport-limiting layer forms a fourth polymeric layer. As described for Example 1, this layer was made of cellulose acetate, and provides substrate transport control, thereby defining the dynamic range of the sensor. Increasing the thickness of the outer cellulose acetate layer decreased the sensor's sensitivity, but increased the apparent K_m . FIG. 3 shows the response of an electrode over-coated with varying amounts of cellulose acetate: (A) 4 μl ; (B) 8 μl ; (C) 28 μl of 0.25% cellulose acetate. Increasing the thickness of the outer cellulose acetate layer from 4 μl (A) of 0.25% cellulose acetate to 8 μl (B) decreased the sensitivity by 50%, but increased the apparent K_m from 1 mM to 2 mM

glucose. The response time (t_{10-90}) for electrodes with an outer cellulose acetate layer of 8 μl of 0.25% was less than 2 minutes.

Example 4

Potential Dependence of the Electrodes

The redox polymer of the electrodes of Example 1, POs-EA, has a formal redox potential of +278 mV (vs. SCE). (Gregg and Heller, 1991, *J Phys. Chem.* 95:5970) FIG. 4 shows the dependence of current density on the applied potential for a glucose electrode of Example 1. Between 0.20 V and +0.35 V (vs. SCE), the current density was independent of applied potential. At applied potentials positive of +0.40 V, a reduction current was no longer observed. At applied potentials more negative than -0.20 V, oxygen reduction effects are observed.

Example 5

Oxygen Dependence of the Electrodes

In nature, the reduced form of an oxidase requires oxygen for reoxidation and production of hydrogen peroxide. FIG. 5 shows the dependence of the current density on the partial pressure of the dissolved oxygen for the electrodes of Example 1. At 37 C, the concentration of oxygen in an air-saturated aqueous solution is about 0.2 mM (See, Hodgman Inc.: *Handbook of Chemistry and Physics*, 44th Ed, The Chemical Rubber Publishing Co., Cleveland, Ohio, 1963, page 1706).

For glucose electrodes, even at very low oxygen concentrations, the current densities were lower only by about 20% than those in oxygen saturated solutions. For oxygen concentrations of 0.04 mM or higher, i.e., in solutions saturated with atmospheric pressure argon containing only 4 volume % oxygen, the current density was independent of the oxygen concentration.

For lactate electrodes, however, at low oxygen partial pressures, the dependence of current density on oxygen concentration was more substantial. This difference is explained by the different immobilization methods for GOx and LOx. The sodium periodate crosslinked GOx layer has lower oxygen permeability than that of the PDI-GOx crosslinked PVI-LOx layer. At high (5 mM) lactate concentrations, the current density became independent of the partial pressure of oxygen only when the volume % of oxygen reached its volume % in air (21%). For lower lactate concentrations, it is expected that the current will be less dependent on oxygen partial pressure as a lower oxygen flux suffices for the quantitative conversion of lactate to hydrogen peroxide and pyruvate.

Example 6

Salt and pH Dependence of the Electrodes

FIG. 6 shows the dependence of current density on the concentration of NaCl for both GOx (A) and LOx (B) electrodes of Example 1. Although the current densities were somewhat higher at low salt concentrations, the electrodes were virtually insensitive to salt concentrations up to 1M NaCl. Increasing the salt concentration from 0.1M to 1M decreased the current density by less than 10%.

FIGS. 7A and 7B show the dependence of the current density on pH for both GOx (FIG. 7A) and LOx (FIG. 7B) electrodes of Example 1. For the glucose electrodes, the current density was near its maximum between pH 4.5 and

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pH 6.5, the plateau matching the pH dependence of GOx activity in solution, which exhibits maximal activity near pH 5.5. For lactate electrodes, the current density reached its maximum at pH 4.0, differing from the pH dependence of L.Ox activity in solution, which exhibits maximal activity at pH 6.5. Both glucose and lactate electrodes were irreversibly deactivated below pH 3.0.

Example 7

Rotation Rate and Temperature Dependence

FIG. 8 shows the dependence of current density on the angular velocity of the rotating electrodes for both GOx (A) and L.Ox (B). The current density was practically independent of rotation rate. For example, in the case of the glucose electrode, increasing the rotation rate from 50 rpm to 1000 rpm increased the current density by less than 5%.

FIGS. 9A and 9B show the dependence of the current density on temperature for both electrodes, FIG. 9A shows data for 5 mM glucose; and FIG. 9B shows data for 5 mM lactate.

Example 8

Long Term Operational Stability

The stability of the current density of the sensors of Example 1 during continuous operation at 37° C. was a strong function of oxidase loading. As shown in FIG. 10A, the higher the GOx loading, the better the operational stability at 37° C. An electrode loaded with 1.3 µg of GOx (a) had an operational half life of 40 hours, while an electrode loaded with 52 µg of GOx (d) maintained 100% of its initial current density for over 100 hours and lost only 10% of its initial current density after 200 hours, and less than 25% after 280 hours. FIG. 11 shows the current response before and after the long term operational stability experiments for glucose electrodes at both high and low loadings. While the electrode with a 1.3 µg GOx loading lost more than 90% of its current density at all glucose concentrations after 100 hours of operation at 37° C., the electrode with 26 µg GOx loading demonstrated no loss of current density at low concentrations, and a loss of only 25% of current density at glucose concentrations greater than 5 mM after 200 hours of operation. Cyclic voltammetry revealed that the electrodes did not lose electroactive osmium throughout the long term stability experiments (FIG. 12), and operational stability was maintained as long as the amount of active enzyme sufficed to oxidize all of the glucose flux.

Similarly, FIG. 10(B) shows that the higher the L.Ox loading, the better the operational stability at 37° C. While the electrode with a 20 µg L.Ox loading lost more than 80% of its current density at all lactate concentrations after 120 hours of continuous operation at 37° C., the electrode with 160 µg L.Ox loading demonstrated no loss of current density at low concentrations, and a loss of only 10% of current density at lactate concentrations greater than 5 mM after 160 hours of operation. Cyclic voltammetry revealed that the electrodes did not lose electroactive osmium throughout the long term stability experiments, and operational stability was maintained as long as the amount of active enzyme sufficed to oxidize all of the lactate flux (data not shown).

Example 9

Selectivity Against Interferents

Interferents such as ascorbate, urate, and acetaminophen are oxidized at PosEA coated carbon electrodes. These

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interferents decrease the catalytic hydrogen peroxide electroreduction current. Nevertheless, no current change was observed with the electrodes of Example 1, upon the addition of urate up to 0.5 mM or acetaminophen up to 1.0 mM, both in the presence and absence of substrate. Ascorbate was electrooxidized only at a negligibly slow rate. For example, at 1 mM glucose, an injection of 0.1 mM ascorbate caused a decrease of less than 1% in the current density, and, at 64 mM glucose, injection of 0.1 mM ascorbate caused a decrease of less than 0.1% in the current density.

Example 10

Preparation and Activity Test for Lactate Oxidase-doped Silica Gel Powder

Preparation of PVI-LOX-doped Silica Gel Powder

0.15 g of tetraethylorthosilicate (TMOS, Cat. # 34,143-6, Aldrich, Milwaukee, Wis.) was added to a small vial. The vial was placed into an ice-bath, and stirred at approximately 600 rpm. 36 mL of 2.44 M HCl was added and the solution was stirred for 10 min. A vacuum was then applied to the vial for an additional 10 minutes to eliminate the methanol produced in the sol process. The vacuum was released, and pH of the solution was adjusted to pH 5.1 by adding 20 mL of 20 mM phosphate buffer (pH 7.4).

In separate small vial, 6 mg of lactate oxidase (L.Ox, from *acrococcus viridans*, 41 units/mg, Cat. #131, Genzyme, Cambridge, Mass.), 180 mL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) buffer solution (pH 7.5), and 20 mL of 20 mg/mL of aqueous poly(1-vinylimidazole) were mixed as described by Timothy J. Ohara, Ravi Rajagopalan and Adam Heller, *Anal. Chem.* 1994, 66, 2451-57. This solution was added to the TMOS-containing solution and was stirred for 1 minute. A vacuum was applied to the stirred mixture until a gel formed. The vacuum was released and the gel was rinsed with 2 mL of distilled water three times. The gel was then soaked in 2 mL of distilled water overnight at 4° C. The water was removed from the vial and the gel was allowed to dry at room temperature overnight. The dried gel was collected and ground to a powder with a mortar and pestle.

Qualitative Activity Test for PVI-LOX-doped Silica Gel Powder

For a qualitative activity test of lactate oxidase-doped silica gel at room temperature, peroxide test strips (Reflectoquant® 16974 Peroxide Test, Merck, Darmstadt, Germany) were used. 1-2 mg of lactate oxidase-doped silica gel powder was applied to the active area of the test strip. 20 µL of 1 M lactate (prepared in phosphate buffer solution, pH 7.4) was applied to the same area of the test strip. The presence of lactate oxidase activity was indicated by the appearance of a blue color on the active area of the strip.

Quantitative Activity Test for PVI-LOX-doped Silica Gel Powder

In the presence of oxygen, a lactate oxidase catalyzes the oxidation of lactate to pyruvate. In the process, hydrogen peroxide is formed stoichiometrically. The enzymatically formed hydrogen peroxide can be quantitatively measured using a peroxidase linked assay system, which causes the formation of a quinoneimine dye. This dye can be measured spectrophotometrically at 564 nm.

Preparation of Solutions for PVI-LOX-doped Silica Gel Powder Activity Test

Reaction mixture 1 was prepared by combining the following solutions:

- 6.0 mL 0.2 M 3,3-dimethylglutarate [$\text{HO}_2\text{CCH}_2\text{CH}_2\text{C}(\text{CH}_3)_2\text{CO}_2\text{H}$]-NaOH buffer, pH 6.5;

- (b) 3.0 mL peroxidase (from horseradish, Sigma Chemical Inc., St. Louis, Mo.), 50 units/mL in water;
 (c) 3.0 mL 15 mM 4-aminoantipyrine in water;
 (d) 3.0 mL 0.5 M substrate; and
 (e) 9.0 mL distilled water.

A stock solution of 0.5% N,N' -dimethylaniline was prepared by mixing 0.2 g into 100 mL of water. A stock solution of the enzyme diluent was prepared by adding 66 mg of FAD into 4 mL of a 10 mM KH_2PO_4 -NaOH buffer solution, pH 7.0. Lactate oxidase solutions were prepared at a concentration of 1 mg/mL in enzyme diluent. A stock solution of enzymatic reaction stopper was prepared by dissolving 0.25 g of dodecylbenzenesulfonic acid sodium salt [$C_{12}H_{25}SO_3Na$] in 100 mL of distilled water. All chemicals were purchased from Aldrich Chemical Company (Milwaukee, Wis.) unless otherwise mentioned.

Activity Test Procedure for PVI-LOx-doped Silica Gel Powder

1.0 mg of lactate oxidase-doped silica gel powder was weighed into a 3 mL cuvette. 100 μ L of distilled water was used to wash the powder down from the inside walls to the bottom of the cuvette. The cuvette was then capped with parafilm and placed in a temperature bath set at 63° C. for the experimental period of time. After this period of time, the cuvette was removed from the bath, and 100 μ L of enzyme diluent was added. The cuvette was then placed into a 37° C. temperature bath for another five minutes.

A solution with a 4:1 ratio of reaction mixture I to 0.5% dimethylaniline was prepared and allowed to equilibrate at 37° C. One milliliter of this solution was added to the cuvette. This mixture was agitated and allowed to react for exactly 10 minutes at 37° C. Immediately after this incubation, 2.0 mL of the reaction stopper were added and the absorbance at 564 nm (As) was measured.

The procedure was repeated, using 1.0 mg of enzyme-free silica gel powder in place of the lactate oxidase-doped silica gel powder. The same procedure was followed thereafter and the blank absorbance (Ab) was measured. Therefore, the net absorbance of the lactate oxidase-doped silica gel powder was determined by $\Delta A (A_s - A_b)$. If A_s was greater than 0.6, the assay was repeated, and the weight of the lactate oxidase-doped silica gel powder was adjusted accordingly. Results for PVI-LOx-doped Silica Gel Powder

FIG. 13 shows the activity decrease of lactate oxidase in solution over time. The enzyme had a half-life of 6.8 minutes at 63° C., which was in agreement with the previously published value (H. Minagawa, N. Nakayama, and S. Nakamoto, 1995, *Biotechnology Letters* 17(9):975).

FIGS. 14A and 14B show the comparison of the enzyme activity loss of PVI-lactate oxidase-doped silica gel powder with that of lactate oxidase-doped silica gel powder at 63° C. FIG. 14A shows PVI-lactate oxidase-doped silica gel. FIG. 14B compares PVI-lactate oxidase-doped silica gel (a) with lactate oxidase-doped silica gel (b). At 63° C., the LOx-doped silica gel powder (b) had the same half-life as that of LOx in solution. However, the PVI-LOx-doped silica gel powder FIG. 14B(n) and FIG. 14A maintained 60% of its initial activity after 900 minutes at 63° C.

FIG. 15 shows the time dependence of the activity loss for lactate oxidase in different environments at 50° C. At this temperature, the PVI-LOx-doped silica gel powder (A) maintained 85% of its initial activity after 120 hours, whereas the LOx-doped silica gel powder (C) had a half-life of only 10 hours. Both the LOx in solution (D) and the PVI-LOx in solution (C) demonstrated a half-life of less than one hour at this temperature.

The PVI-LOx-doped silica gel powder maintained 100% of its initial activity after 11 days of soaking in water at room temperature.

Example 11

Preparation and Activity Test for Glucose Oxidase-doped Silica Gel Powder

Preparation of GOx-doped Silica Gel

1.527 g of tetramethylorthosilicate (TMOS) was added to a small vial. The vial was placed into an ice-bath, and the solution was stirred at approximately 600 rpm. 360 mL of 2.44 mM HCl was added and the solution was stirred for 10 min. A vacuum was applied to the vial for an additional 10 minutes to eliminate the methanol produced in the sol process. The vacuum was released and the pH of the solution was adjusted to pH 5.1 by adding 100 μ L of 20 mM phosphate buffer (pH 7.4). In a separate small vial, 67 mg of glucose oxidase (GOx from *Aspergillus niger*, Cat. # G-7141, 183 units/mg, from Sigma, St. Louis, Mo.) and 540 mL of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (pH 7.5) were mixed. This solution was added to the TMOS-containing solution and was stirred for 1 minute. A vacuum was applied to the stirred mixture until a gel formed. The vacuum was released, and the gel was allowed to dry overnight at room temperature. The dried gel was first collected and then ground to a powder with a mortar and pestle.

Qualitative Activity Test for GOx-doped Silica Gel

For a qualitative activity test of glucose oxidase-doped silica gel at room temperature, peroxide test strips (Reflectoquant® 16974 Peroxide Test, Merck, Darmstadt, Germany) were used. 1-2 mg of glucose oxidase-doped silica gel powder was applied to the active area of the test strip. 20 μ L of 1 M glucose (prepared in phosphate buffer solution, pH 7.4) was applied to the same area of the test strip. The presence of glucose oxidase activity was indicated by the appearance of a blue color on the active area of the strip.

In order to test the activity of the glucose oxidase-doped silica gel powder at higher temperatures, the powder was immobilized onto a glass slide according to the following procedure. 26 mg of Bermocoll® E1IM-100 (Berol Nobel AB, Stenungsund, Sweden), 1 mL of cyclohexanone and 1 mL of water were mixed in a small vial. The vial was heated and agitated over a flame for 5 minutes. The solution was allowed to settle and the white micelle fraction was collected. 90 μ L of the micelles and 10 mg of the glucose oxidase-doped silica gel powder were mixed and then stirred for 5 minutes. 10 μ L of the mixture was applied to a glass slide and allowed to dry overnight. The glass slide was immersed in a temperature bath for 5 minutes at the experimental temperature. The slide was removed and allowed to dry. 10 μ L of 2 M glucose was applied to the immobilized micelles. The active area of the peroxide testing strip was placed on top of the immobilized micelle area. The appearance of a blue color on the strip indicated the presence of glucose oxidase activity.

Quantitative Activity Test for GOx-doped Silica Gel

The quantitative activity test of glucose oxidase was performed exactly as that for lactate oxidase except a 1 M glucose solution was used in place of the 1 M lactate solution and glucose oxidase-doped silica gel powder was used in place of lactate oxidase-doped silica gel powder. Qualitative Results for GOx-doped Silica Gel

The glucose oxidase-doped silica gel powder retained activity after immersion in water for 5 minutes at 40, 50, 60, 70, 80, and 92° C., while similarly immobilized glucose oxidase in DAEA Dextran, Heparin, 1 acitol, Galgatus, and Dextran Sulfate showed no activity retention at temperatures higher than 70° C.

The glucose oxidase-doped silica gel powder was then tested for activity at 92° C. for longer time periods. Activity

was retained for up to 20 minutes, after which, the micelles washed off of the slides. At 75° C., activity was retained for over 60 minutes.

Quantitative Results for GOx-doped Silica Gel

FIG. 16 shows the comparison of the enzyme activity loss of glucose oxidase-doped silica gel powder (B) with that of glucose oxidase in solution (A) at 65° C. At this temperature, the GOx-doped silica gel powder (B) maintained 60% of its initial activity after 1200 minutes, while the half-life of GOx in solution (A) was 6.5 minutes, as shown in the figure inset.

Example 12

Preparation and Activity Test for PEI-LOx-doped Silica Gel

Polyethyleneimine (PEI) is chemically similar to PVI and has been shown to stabilize enzymes. PEI-LOx-doped silica gel powder was prepared according to the same procedure as that for PVI-LOx-doped silica gel powder. FIG. 17 shows the activity loss over time of PEI-LOx-doped silica gel powder at 63° C. After 900 minutes, the PEI-LOx-doped silica gel powder maintained 18% of its initial activity.

Example 13

Preparation and Activity Test of PVI-LOx Adsorbed onto Colloidal Silica

PVI-LOx was also adsorbed onto colloidal silica according to the following procedure. 1.69 mg of LOx was dissolved in 23 μ L of 20 mg/mL of PVI. This solution was mixed with 33 μ L of colloidal silica (34 wt. % suspension in water, Aldrich, Milwaukee, Wis.). The resulting solution was allowed to dry. The solid was first collected and then ground to a powder in a mortar and pestle. The PVI-LOx adsorbed silica had the same half-life as LOx in solution at 63° C.

Example 14

Layered Sensor with Sol-Gel Silica Immobilized GOX Particles in Silicone

A 0.29 mm polyimide insulated gold wire (polyimide insulation thickness 0.02 mm, gold wire OD 0.25 mm) was etched galvanostatically in a KCN solution as described by E. Csoregi et al., *Analytical Chemistry* 66:3131-3138, 1994, to form about 100 μ m deep recess. The following sequence of droplets were deposited in the recess to form a four-layered structure. The innermost layer contained a hydrogen peroxide-sensing soybean peroxidase and redox polymer. The second layer was a hydrogen peroxide permeable insulating layer. The third layer was a silicone layer in which sol gel silica-immobilized glucose oxidase containing particles were dispersed. The fourth, cellulose acetate layer was oxygen permeable and glucose flux reducing.

To form these four layers on the electrode, the following procedure was used. The redox polymer solution prepared as described for Example 1 was used at a concentration of 10 mg/mL. Soybean peroxidase (SBP) in deionized water was used at a concentration of 10 mg/mL. PEGDGE was used at 5 mg/mL. The redox polymer, SBP, and PEGDGE solutions were mixed at a 2:2:1 ratio. Ten droplets of about 5 nL each of the mixed solution were sequentially deposited within the recess to form the sensing layer, which was cured at 55° C. for 20 minutes.

The solution from which the second layer, the hydrogen peroxide permeable membrane, was cast, consisted of 0.5%

weight % cellulose acetate (40% acetylated) in cyclohexanone. One 5 nL droplet of this solution was applied to the cured sensing layer in the recess.

The hydrogen peroxide generating enzyme layer (third layer) was made of 0.5 parts of the sol-gel immobilized glucose oxidase of Example 15, 1 part of Dow Corning 3-5025 silicone water based elastomer that was thoroughly mixed and dispersed in 9 parts of water by grinding in an agate mortar for 30 minutes. Three drops of this mixture were applied on the cellulose acetate layer, and cured at 50° C. for ten minutes.

The fourth layer was made of 2 weight % cellulose acetate (40% acetylated) also containing 1.6 weight % TWEEEN 60 in cyclohexanone, of which one drop was applied onto the third layer.

When the electrode, produced as described above, was poised at -0.045V (SCE) in a phosphate buffer solution at pH 7.0 (20 mM phosphate, 0.14 M NaCl), the current in the absence of glucose was 0.1 nA. When the glucose concentration was brought to 5 mM, the current increased in less than 20 seconds to 3.6 nA. Further increases of the concentration to 10 mM resulted in respective currents of 6.6 nA and 8.5 nA.

Example 15

Two Layered Glucose Electrode

Recessed palladium electrodes were prepared as described by E. Csoregi et al., *Analytical Chemistry* 67:1240, 1995, for gold electrodes, except that the solution in which the palladium was etched under galvanostatic control was not a potassium cyanide solution, but 6M Hcl. The recess formed was about 100 μ m deep. Aqueous solutions of 10 mg/mL soybeanperoxidase, 10 mg/mL POs-EA and 5 mg/mL PEGDGE were prepared.

In a small vial, 20 μ L of the peroxidase solution, 20 μ L of the POs-EA solution, and 10 μ L of the PEGDGE solution were mixed. 5 nL of this mixed solution were applied to the palladium surface. The solution was allowed to dry, and the application and drying steps were repeated seven times. The electrode was allowed to cure overnight at room temperature.

In a small vial, 0.1 g of Dow Corning Water Based Elastomer Silicone (#3-5025, Lot #LL059026) and 0.9 g of water were thoroughly mixed. 0.95 μ L of this mixture and 0.05 μ L of glucose oxidase-doped silica gel powder were mixed. 5 nL of this mixture were applied on top of the "wired" soybean peroxidase layer of the recessed palladium electrode. The electrode was then cured for ten minutes at 50° C.

The electrode was poised at -0.045 V (SCE) in a phosphate buffer solution at pH 7.0 (20 mM phosphate, 0.14 M NaCl) and its current was measured as a function of the glucose concentration in the solution. The apparent K_m of the electrode was 5 mM glucose, i.e., half of the maximum current was reached at 5 mM glucose. The response times, i.e., the rise of the current from 10 to 90% of the maximum current, were less than one minute. The sensitivity of the electrode was 0.8 nA/nM glucose.

We claim:

1. An electrochemical hydrogen peroxide sensor comprising:
 - a) an electrode; and
 - a) a thermostable peroxidase immobilized on and electrically connected to the electrode;

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the sensor being capable of operation at 37°C. with less than 2% loss of sensitivity per hour of continuous operation.

2. The hydrogen peroxide sensor of claim 1, wherein the thermostable peroxidase is soybean peroxidase.

3. The hydrogen peroxide sensor of claim 1, further comprising a redox polymer disposed on the electrode and coupled to the thermostable peroxidase.

4. The hydrogen peroxide sensor of claim 3, wherein the thermostable peroxidase is crosslinked to the redox polymer.

5. The hydrogen peroxide sensor of claim 3, wherein the redox polymer comprises an osmium cation.

6. The hydrogen peroxide sensor of claim 5, wherein the osmium cations are coordinatively bound to the redox polymer.

7. The thermostable peroxidase of claim 1, wherein the peroxidase is non-leachable.

8. The hydrogen peroxide sensor of claim 1, further comprising a biocompatible layer disposed over the thermostable peroxidase and the electrode.

9. The hydrogen peroxide sensor of claim 1, further comprising a second enzyme capable of catalyzing the production of hydrogen peroxide in the presence of an analyte.

10. The hydrogen peroxide sensor of claim 9, wherein the second enzyme is disposed on the electrode but electrically insulated from the electrode.

11. The hydrogen peroxide sensor of claim 9, wherein the second enzyme is insulated from the electrode by the intrinsic protein structure of the second enzyme.

12. The hydrogen peroxide sensor of claim 9, wherein the second enzyme is insulated from the electrode by a hydrogen peroxide-permeable membrane or polymer.

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13. The hydrogen peroxide sensor of claim 1, wherein the sensor is capable of operation at 50° C. with less than 5% loss of sensitivity per hour of continuous operation.

14. The hydrogen peroxide sensor of claim 13, wherein the sensor is capable of operation at 50° C. with less than 2% loss of sensitivity per hour of continuous operation.

15. The hydrogen peroxide sensor of claim 1, wherein the sensor is adapted for in vivo use.

16. The hydrogen peroxide sensor of claim 1, wherein the sensor is capable of operation at 37° C. for five days with less than 10% loss of sensitivity.

17. The hydrogen peroxide sensor of claim 1, wherein the sensor is capable of operation at 37° C. with less than 2% loss of sensitivity per hour of continuous operation with intermittent measurements of analyte concentration.

18. The hydrogen peroxide sensor of claim 1, wherein the sensor is adapted for subcutaneous implantation.

19. An electrochemical hydrogen peroxide sensor comprising:

an electrode; and

a thermostable peroxidase immobilized on and electrically coupled to the electrode;

wherein, in the presence of hydrogen peroxide, the thermostable peroxidase generates an electric signal.

20. An electrochemical sensor comprising:

an electrode;

a thermostable peroxidase disposed on the electrode; and

a redox polymer disposed on the electrode configured and arranged to couple the peroxidase to the electrode.

* * * * *

EXHIBIT 2

Glucose ENFET doped with MnO₂ powder

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Abstract

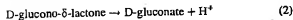
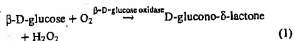
In this study, a glucose-sensitive enzyme field-effect transistor (ENFET) coimmobilized with glucose oxidase (GOD) and manganese dioxide (MnO₂) have been investigated. The biomembrane of the ENFET was immobilized on the amorphous tin oxide/indium tin oxide glass structure extended sensitive gate, which used as a disposable transducer of a glucose biosensor. MnO₂ was used as a catalyst which can catalyze the hydrogen peroxide and produced H₂O and O₂. Coimmobilization of glucose oxidase and manganese dioxide was found to be useful for extending the dynamic measured range of glucose concentration to 360 mg/dl (eq. 20 mM). The result shows that the dynamic range of the output signal is strongly dependent on pH value of measuring environments, and the measurement in the alkali buffer solutions shows a higher response and wider dynamic range. Additionally, the different immobilized layers of MnO₂ have been studied. The MnO₂, which be immobilized in outer cross-linking layer of bovine serum albumin, shows better results than immobilized in GOD layer or glutaraldehyde covalent layer. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Extended gate field effect transistor (EGFET); Enzyme field effect transistor (ENFET); Amorphous tin oxide; Glucose oxidase (GOD); Manganese dioxide (MnO₂)

1. Introduction

Since the first reported enzyme biosensor (ENFET) based on ion-sensitive field effect transistors (ISFETs) [1], substantial research efforts were undertaken to improve the performance characteristics of the ENFETs developed. Until now, there are almost two dozen papers dealing with glucose ENFETs which suffer from many problems [2].

Normally glucose oxidase hydrolyzes glucose according to the following reactions:



ISFET sensors measure the glucose concentration by detecting the pH variation due to the hydrogen ions that are

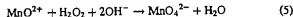
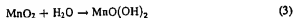
generated by the dissociation of gluconic acid. However, because of the low dissociation constant ($pK_a \approx 3.8$) [3], ISFET glucose sensors show low sensitivities. Generally, the sensitivities at the physiological pH value are limited to only some millivolts per decade [4]. Hence the ISFET drift, which is an inherent characteristic of ISFETs, becomes a important topic. The glucose concentration in human blood is normally about 5 mM, reaching 20 mM and more for diabetics. However, the concentration of oxygen, does not exceed 0.5 mM. Because of the unfavourable concentration ratio of glucose and oxygen in real blood, the dynamic range of the biosensor is usually limited by oxygen and dose not exceed several mM. Since, the oxygen in the sensor membrane is consumed by the enzyme reaction, the oxygen concentration is needed high enough for a better linearity between output voltage and the glucose concentration. Moreover, the hydrogen peroxide, one of the by-products of the glucose oxidation, acts an inhibitor of glucose oxidase which causes the lower sensitivity and bad repeatability in the steady measurement system of glucose ENFET.

Sudoh et al. employed pre-electrolysis method to enrich the oxygen of the glucose solution, which the oxygen is generated by electrolysis of the solution before monitoring

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[5]. The output signal was linearly proportional to the glucose concentration up to 500 mg/dl by the supplement of oxygen generated by electrolysis of the solution. Seo et al. and Lee et al. employed a Pt electrode actuator on the ISFET sensitive gate to electrolyze the hydrogen peroxide [2,6]. The sensor with the Pt electrode actuator exhibits a wide dynamic range that from 1 to 10 mM. Saito et al. used an external BSA membrane, which is highly cross-linked by glutaraldehyde, to restrict glucose diffusion to expand the measuring range and make a stable response in a low buffer capacity solution [7]. The sensor outputs shows a good linear relationship with up to 300 mg/dl glucose concentration. Shul'ga et al. added the potassium ferricyanide, which used as an oxidizing substrate in the biocatalytic oxidation of glucose, into the phosphate and TRIS buffer to perform the glucose ENFET measurement [8]. Depending on the concentration of ferricyanide the glucose ENFET shows a 10–100 times increase of the biosensor response and a substantial extension of its dynamic range.

In this paper a glucose sensor based on H^+ -ion-sensitive field effect transistor (ISFET) has been realized in combination with a MnO_2 -doped glucose oxidase membrane. Zheng and Guo brought forward the following procedures and reactions of H_2O_2 catalyzed by MnO_2 [9]:



Where MnO_2 was used as a catalyst which can catalyze the hydrogen peroxide and produce H_2O and O_2 . In addition to the reduction of H_2O_2 concentration in the biolayer, the product, oxygen, can be recycled for glucose oxidation reaction. The MnO_2 doping position and the pH value of working have been investigated.

2. Experimental

2.1. Chemicals and materials

The β -D-glucose oxidase (GOD) EC 1.1.3.4 from *Aspergillus niger*, bovine serum albumin from Serva and γ -aminopropyl triethoxysilane (3-APTS, 99%) were purchased from Sigma. Glutaraldehyde (GA, 25% aqueous solution) was purchased from Acros Organics. Manganese dioxide powder (99.9%) was obtained from Tekstart (Hsinchu, Taiwan). All other reagents were in reagent grade and were used without further purification. Distilled water was used for all the electrolytes and the buffer solutions. Tin oxide thin films were formed by the RF sputtering system (tin oxide target, 99.9%) at a substrate temperature of 150°C. The ITO glasses (50–100 Ω /sq; ITO coating thickness, 230 Å) were supplied by the Wintek Corporation.

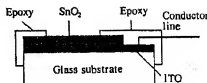


Fig. 1. Cross-section of SnO_2 /ITO glass sensing structure.

2.2. Sensor Fabrication

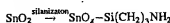
2.2.1. Solid-state part

In this study, the glucose ENFET is based on a separative extended gate ISFET (EGFET) structure. The sensitive part of the separative EGFET is shown as Fig. 1. The SnO_2 thin film was deposited by using sputtering method with a thickness of 2000 Å. Before the glass was deposited SnO_2 , it was washed in methyl alcohol and DI water for 20 and 10 min, respectively. The SnO_2 /ITO glass EGFET shows a linear pH response about 57 mV/pH between pH 2.4 and pH 11.2 [10].

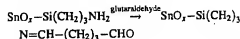
2.2.2. Enzyme Immobilization

The procedure for preparation of separative structure of ENFET is as follows.

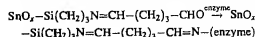
1. Cleaning: The separative structure of EGFET was cleaned by distilled water.
2. Silanization: There is no amino group on our sensitive film, so we use 3-APTS to modify tin oxide (SnO_2) substrate. The procedure is represented as follow [11,12]:



3. Activation by glutaraldehyde: Glutaraldehyde (1%) is also used extensively to immobilize enzyme molecules onto a carrier substance bearing amino group. The procedure is represented as follow:



4. Coupling of the enzyme and cross-linking: The GOD (40 mg) was dissolved in 1 ml of a 0.1 M K-P buffer solution (pH 7.0). A 1.5 μ l part of the solution was cast onto the gate region and then addition of 1 μ l of the glutaraldehyde was followed to chemically cross-link the membrane. The procedure is represented as follow:



5. The outer BSA membrane doping with MnO_2 : An amount of 10 mg MnO_2 was dissolved in 300 mg/dl BSA and 6% glutaraldehyde (1:1) solution. A 1 μ l part of the solution was cast onto the enzyme membrane.

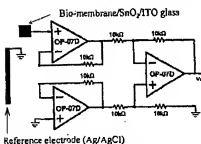


Fig. 2. Separative extended ENFET measurement circuit with instrumentation amplifier LT1167.

In the study of the effect of MnO_2 doping position, the outer BSA membrane was not immobilized, and the MnO_2 was doped in activation or GOD with 10 mg/ml MnO_2 .

2.3. Measurement

A readout circuit based on an instrumentation amplifier LT1167 is shown in Fig. 2. The measurement configuration consists of a separative extended gate with biomembrane and the Ag/AgCl reference electrode. The instrumentation amplifier, LT1167, was a transducer and the small output voltage will depend on the pH value. HP3478A and HP VEE program were designed and used as an Y-T recorder to record the voltage variation with time. All measuring temperature of our experiments are in 25°C , 5 mM phosphate-KOH buffer.

3. Results and discussion

3.1. Glucose ENFET response

In this study, a separative sensitive gate of biomembrane/ SnO_2 /ITO glass structure was used as a disposable biochemical transducer. This structure has advantages of light insensitivity, easier fabrication processes than traditional ISFET and lower cost than SOS structure ISFETs or silicon based EGFET [10]. Fig. 3 shows the pH response of separative sensitive structure with biomembrane of sensitivity 58.3 mV/pH between pH 2 and 10.

Figs. 4 and 5 show typical time response curve for the glucose ENFET without and with the outer BSA membrane. The glucose ENFET was immersed in blank buffer solution for 1 min and then immersed in glucose solution. As the ENFET are measured in blank buffer, it shows a drift that not exceeds to 1 mV for 1 min. The glucose ENFETs without and with BSA membrane show response time of 5 and 12 min, respectively. The glucose ENFET, which has outer MnO_2 -doped BSA membrane, shows a good linearity up to 360 mg/dl, because the O_2 concentration in biomembrane was greatly rose by MnO_2 catalyzing H_2O_2 . The relationship between the sensor output and glucose concentration is shown in Fig. 6.

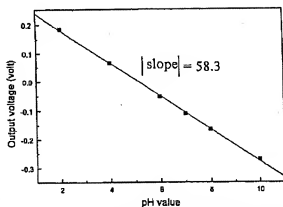


Fig. 3. Output voltage vs. pH value for the biomembrane/ SnO_2 /ITO glass sensing gate connected with instrumentation amplifier LT1167.

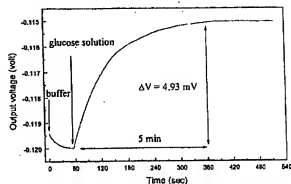


Fig. 4. Response of the separative structure of the ENFET without outer BSA membrane to detect 40 mg/dl glucose in pH 7.2 buffer solution. MnO_2 was immobilized in GOD layer.

Saito et al. employed the glucose ENFET with external BSA membrane, which is very similar our device, shows a good linear relationship with up to 300 mg/dl glucose concentration [7] which the experiments are performed in a

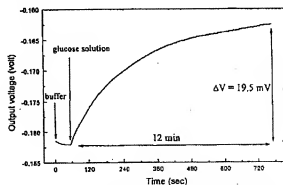


Fig. 5. Response of the separative structure of the ENFET with outer BSA membrane to detect 45 mg/dl glucose in pH 8.5 buffer solution. MnO_2 was immobilized in BSA layer.

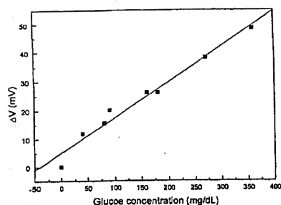


Fig. 6. Calibration curve for the glucose ENFET with outer MnO_2 -doped BSA membrane. Sensors were measured in pH 8.1, 5 mM buffer solution.

stirring status. But, all the measurements in this paper are performed in a steady status. Fig. 7. shows the sensor response for sensors with non-doped MnO_2 outer BSA layer and MnO_2 -doped BSA layer. The results show that sensors with MnO_2 -doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer. The sensors of non- MnO_2 -doped show a high response in lower glucose concentration, but very low response in high glucose concentration.

3.2. Effect of pH on the ENFET response

According to the report of Zheng and Guo, in the experiment of potentiometric determination of hydrogen peroxide at MnO_2 -doped carbon paste electrode, while the pH changed in the range 7.0–8.0, the potential response increased with increasing pH [9]. The results may be related to the enhancing of oxidizing ability of H_2O_2 when pH changed in this range. For pH values in the range of 8.0–9.0, the

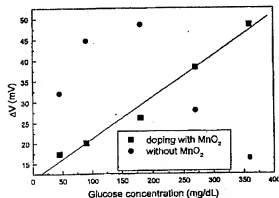


Fig. 7. Calibration curve for the glucose ENFET sensors with (●) non-doped MnO_2 outer BSA layer and (■) MnO_2 -doped BSA layer. Sensors were measured in pH 8.1, 5 mM buffer solution.

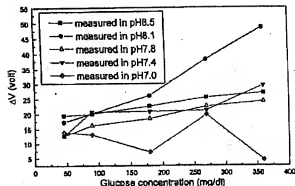


Fig. 8. Effect of pH on the glucose ENFET with outer MnO_2 -doped BSA membrane. The curves correspond to different pH values of measured environments: (■) pH 8.5; (●) pH 8.1; (▲) pH 7.8; (▼) pH 7.4; (◆) pH 7.0.

response was almost constant. In our research, the effect of pH on the glucose ENFET response shows the best results of sensitivity and linearity that are shown in Fig. 8. As the GOD catalyzes glucose, the actual pH value of the ENFET biomembrane is lower than the pH value of buffer. The output signal of the measurement in pH 8.5 is lower than that of in pH 8.1 which is caused by that the activity of the GOD is bad in alkali [13,14]. In addition, the results measured in lower pH environments show a bad linearity which is caused by that the MnO_2 shows lower catalysis ability in acid.

3.3. Effect of MnO_2 doping position

As mention before, while the pH changed in the range 7.0–8.0, the catalysis ability of MnO_2 increased with increasing pH value. However, the actual pH value is different in individual biomembrane layer as the ENFET dips into glucose solution. The GOD layer shows the lowest pH that caused by the glucose catalyzed and producing H^+ ion. The H^+ will diffuse into the activation layer and outer layer, which close to the SnO_2 sensitive film and pH-buffer

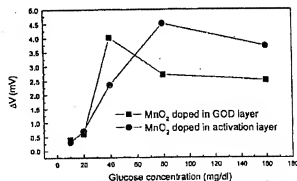


Fig. 9. Glucose concentration response of the ENFET that MnO_2 -doped in the GOD layer and in the activation layer, respectively.

solution, respectively. By the effect of the carrier-mediated transport of protons, the outer membrane will show the highest pH value, which is better suitable condition for the reaction between MnO_2 and H_2O_2 . As the results shown above, the glucose ENFET that biomembrane with outer MnO_2 -doped BSA membrane has a linear dynamic range to 360 mg/dl. Fig. 9 shows the glucose response of MnO_2 -doped in the activation layer and the GOD layer. The responses are limited in the high glucose concentration, which caused by that the reaction between MnO_2 and H_2O_2 was blocked in acid environments, especially the response of the device was MnO_2 -doped in enzyme layer.

4. Conclusions

A glucose ENFET based on a new principle, in which the biomembrane was doped with MnO_2 powder. MnO_2 was used to perform the catalysis of hydrogen peroxide (one of the by-products of glucose oxidation), was proposed and its characteristics were investigated. The sensor shows a wide dynamic range to the glucose concentration of 360 mg/dl. Both the pH value of buffer solution and MnO_2 -doped position affect the response of the glucose ENFET. For the MnO_2 -doped effect, both the responses of MnO_2 -doped in the activation layer and the GOD layer are limited in the high glucose concentration, which is caused by that the reaction between MnO_2 and H_2O_2 blocked in acid environments, especially the response of the device that MnO_2 -doped in the enzyme layer. For the effect of pH value of buffer solution, the glucose ENFET, which MnO_2 -doped in the outer BSA layer, measured in pH 8.1 has the largest response and the widest dynamic range in our experiments. In addition, the sensors with MnO_2 -doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer.

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